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Substrate Recognition by the 2-Hydroxycarboxylate Transport Proteins

Michael Bandell

Voor André, Marjan en Soesja

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RIJKSUNIVERSITEIT GRONINGEN

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Michael Bandell

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Prof. Dr. B. Poolman

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Voorwoord

Met het schijven van dit proefschrift rond ik een periode af waarin ik met veel plezier mijn interesse voor de moleculaire biologie heb kunnen botvieren. Het idee om na mijn studie te gaan promoveren kwam tijdens een afstudeerproject aan het Public Health Research Institute in New York. Aldaar werd ik aangestoken door het enthousiasme waarmee in dat instituut moleculair biologisch onderzoek werd gedaan. I want to thank David Perlin and Donna Seto-Young for the excellent times I had in New York and their great enthousiasm towards science. You both played a big part in my decision to continue in science and get my PhD.

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Tenslotte. Lieve Bella, jij hebt het hele circus van dichtbij meegemaakt en jouw steun en liefde is van onschatbare waarde geweest. Ik hou ontzettend veel van je. You and me, wij zullen samen nog ver komen!

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The 2-Hydroxycarboxylate Transporter Protein Family.

STRUCTURE-FUNCTION RELATIONSHIPS

Michael Bandell and Juke S. Lolkema

INTRODUCTION

All living cells contain a large number of integral membrane proteins and protein complexes. One of their main functions is to allow the controlled transmembrane passage of membrane impermeable solutes. All known and putative transport proteins have recently been classified in different families based on their sequence similarities, which led to the identification of over 250 transporter families (1). Secondary transporters represent the largest functional category of transporters, comprising 78 families. To drive transport, these proteins use the free energy stored in electrochemical ion and/or solute gradients across the membrane.

The 2-hydroxycarboxylate transporter (2-HCT) family is a group of homologous secondary transporters. These proteins are found exclusively in bacteria and share a specificity for substrates containing a 2-hydroxycarboxylate motif (Figure 1). All characterised 2-HCT transporters catalyze the uptake of either citrate or (S)-malate coupled to either a proton or a sodium ion gradient or both to drive uptake. In addition, some of the 2-HCT transporters exchange external citrate or (S)-malate for internal lactate, the metabolic breakdown product of citrate and (S)-malate in some organisms. Exchange between internal and external substrates is a well known phenomena in mitochondria but has only recently been discovered in bacteria (for reviews see refs 2-4). The exchangers of the 2-HCT family are involved in the generation of metabolic energy for the cell. Exchange creates a membrane potential due to the difference in charge between the divalent substrates citrate or (S)-malate and the monovalent breakdown product lactate (5-8). It was shown that this membrane potential, in combination with the consumption of a cytoplasmic proton during the breakdown of citrate or (S)-malate, results in a

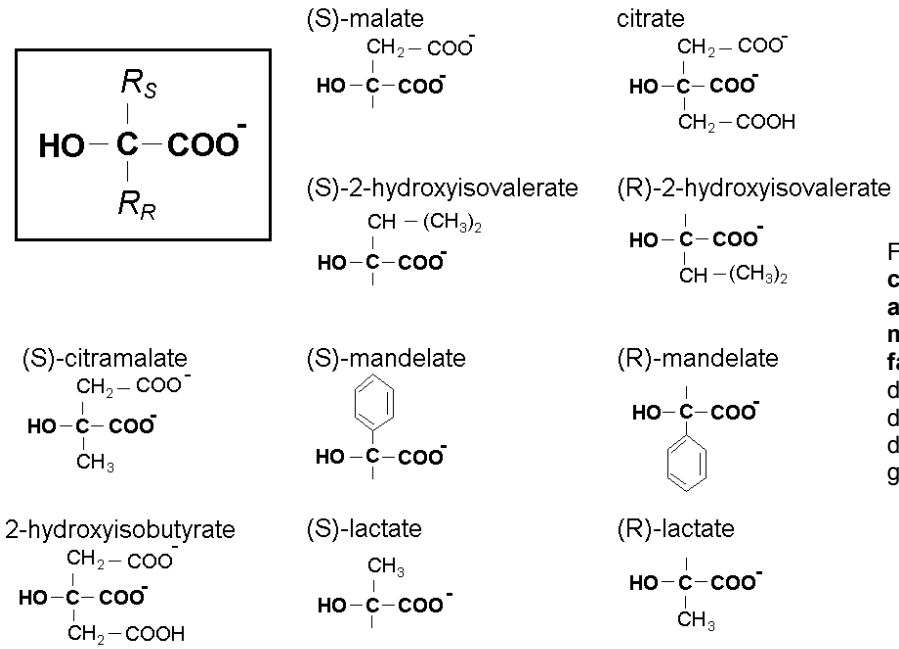

Figure 1. **The 2-hydroxy-carboxylate motif (inset) and some substrates of members of the 2-HCT family.** The R groups are defined as R_R and R_S to discriminate between the different positions of the R groups in chiral compounds.

electrochemical proton gradient that is sufficient to drive ATP synthesis (6). Other than playing a role in the energy status of the cell, the exchangers have been shown to play a role in the recovery from acid stress and resistance against lactate toxicity (9,10).

The ability to exchange substrates that differ in structure and charge implies specific requirements for the substrate binding site. In this review we report the results of recent studies on the structural and functional properties of the 2-HCT transporters and focus on the mechanism behind substrate recognition.

CURRENT MEMBERS

Screening of the sequence databases at the websites of the NCBI and TIGR institutes (http://www.ncbi.nlm.nih.gov and http://www.tigr.org), using the BLAST facility (11), revealed a total of 18 2-HCT family members, all of bacterial origin. For some members several highly homologous variants exist (>95% identical residues), leaving 11 typical proteins in the family (Table 1). Of the 11 typical proteins 7 have been functionally characterized. The uncharacterised members of the family were termed after their closest homolog in the 2-HCT family. A four letter extension was used to discriminate between the different proteins. A hypothetical 2-HCT transporter from *Treponema denticola* that is equally similar to all other family members was arbitrarily termed $CitY_{TRDE}$.

The characterised members of the 2-HCT family all catalyze unidirectional uptake of citrate or (S)-malate or both. Transport is coupled to cotransport of sodium ions or protons or both protons and sodium ions (Table 1). It should be noted, however, that although some members were indicated to be sodium symporters the role of protons was not studied in these cases. In addition to unidirectional transport, CitP of *Leuconostoc mesenteroides* ($CitP_{LEME}$) and MleP of *Lactococcus lactis* ($MleP_{LALA}$) are able to exchange respectively external citrate or (S)-malate for internal lactate, the metabolic end product of citrate and (S)-malate degradation in lactic acid bacteria (5-8). This has been termed precursor-product exchange (2). Precursor-product exchange by CitP and MleP was found to be much faster than unidirectional uptake citrate or (S)-malate and is thought to be the relevant mode of transport in the presence of internal lactate (6,7). Exchange has an energetic advantage over unidirectional transport. Uptake of the external substrate is driven by the chemical concentration gradients of the internal product and the external substrate thus there is no loss of metabolic energy stored in the electrochemical

Table 1. **Members of the 2-hydroxycarboxylate family**

Protein	Organism	Function		Accession no.	Reference
		Unidir. transport[d]	Pre-pro exchange[d]		
$CitP_{LEME}$[a]	*Leuconostoc mesenteroides*	Citrate/H^+	Citrate-lactate	L29572	(5,7,8,29)
$MleP_{LALA}$	*Lactococcus lactis*	(S)-Malate/H^+	(S)-Malate-lactate	X75982	(5,6)
$CitS_{KLPN}$	*Klebsiella pneumoniae*	Citrate/H^+/Na^+	None	P31602	(5,12,31)
$CitC_{SADU}$[a]	*Salmonella dublin*	Citrate/Na^+ [b]	Unknown	P31603	(16)
$CitS_{VICH}$	*Vibrio cholerae*	Unknown	Unknown	AE004164	(50)
$CitY_{TRDE}$	*Treponema denticola*	Unknown	Unknown	[c]	[c]
$MaeP_{STBO}$[a]	*Streptococcus bovis*	(S)-Malate/H^+	Unknown	U35658	(14)
$MaeP_{STPY}$[a]	*Streptococcus pyogenis*	Unknown	Unknown	[c]	[c]
$MaeN_{BAAN}$	*Bacillus anthracis*	Unknown	Unknown	[c]	[c]
$MaeN_{BASU}$ (YufR)	*Bacillus subtilis*	(S)-Malate/Na^+ [b]	Unknown	Z93937	(15,51)
$CitZ_{BASU}$ (YxkJ)	*Bacillus subtilis*	Citrate/H^+; Malate/H^+	Unknown	D83026	(51, [e])

[a]One representative, of 2 or more highly homologous proteins (> 95 % identical residues), is shown. Variants to $CitP_{LEME}$ were found in *Lactococcus lactis*, *Leuconostoc lactis* and *Leuconostoc paramesenteroides*. Variants to $CitC_{SADU}$ were found in *Salmonella pullorum* and *Salmonella enteritidis*. A variant to $MaeP_{STBO}$ was found in *Enterococcus feacalis*. A variant to $MaeP_{STPY}$ was found in *Streptococcus pyogenis* Manfredo. [b]The translocation of H^+ was not studied. [c]Preliminary sequence data was obtained from The Institute for Genomic Research website http://www.tigr.org. and the National Centre for Biotechnology Information at website http://www.ncbi.nlm.nih.gov/. [d]Unidir., unidirectional; Pre-pro, precursor-product. [e]B. Krom, personal communication.

proton or sodium ion gradients. In fact, due to the difference in charge between the monovalent lactate and the divalent citrate/(S)-malate the exchange creates a membrane potential and thus metabolic energy (5-8). Up to now precursor-product exchange has been observed only for $CitP_{LEME}$ and $MleP_{LALA}$. It should be noted however that only CitS of *Klebsiella pneumoniae* ($CitS_{KLPN}$) was actually shown to be unable to catalyze exchange between citrate and lactate (5) or any of the metabolic breakdown products of citrate fermentation in *K. pneumoniae* (12). For all other 2-HCT proteins it remains to be investigated whether these transporters can catalyze precursor-product exchange.

PHYLOGENETICS AND SEQUENCE ALIGNMENT

The 2-HCT proteins range in size from 425 to 450 amino acid residues. A multiple sequence alignment of 2-HCT proteins with less than 95% identical residues was generated, using Clustal X (13). Phylogenetic analysis suggests three clusters of related proteins (Figure 2). These clusters may define functionally separate groups of proteins within this family. Consistent with their specific function as precursor-product exchangers $MleP_{LALA}$ and $CitP_{LEME}$ are located together in one cluster. They share 45% identical residues and have 25-34 % identical residues with the other 2-HCT proteins. The second cluster consists of $MaeP_{STBO}$, $MaeP_{STPY}$, $MaeN_{BASU}$ and $MaeN_{BAAN}$. These proteins share a minimum of 49 % identical residues and have 26-36 % identical residues with the other 2-HCT transporters. The two characterised transporters in this cluster, $MaeP_{STBO}$ (14) and $MaeN_{BASU}$ (15) both transport (S)-malate but not citrate which suggests a common specificity for (S)-malate in this cluster. Interestingly, $MaeN_{BASU}$ uses sodium as coupling ion whereas $MaeP_{STBO}$ uses protons which indicates that the coupling ion specificity is not necessarily conserved within the clusters. The third cluster consists of $CitS_{KLPN}$, $CitC_{SADU}$ and $CitS_{VICH}$. $CitS_{KLPN}$ and $CitC_{SADU}$ both transport citrate but not (S)-malate (5,16). The uncharacterised $CitS_{VICH}$, which shares 66 % identical residues with $CitS_{KLPN}$ and only 25-35 % with the other 2-HCT proteins, is likely to have the same substrate specificity. $CitZ_{BASU}$ and $CitY_{TRDE}$ do not belong to any of the clusters and branch off close to the centre of the phylogenetic tree indicating that they have diverged from the other DNA sequences early in evolution (17). They share 25-39 % and 29-35 % identical residues with the other family members, respectively. In conclusion the clusters seem to represent subfamilies of (i) exchangers, (ii) malate transporters, (iii) citrate transporters.

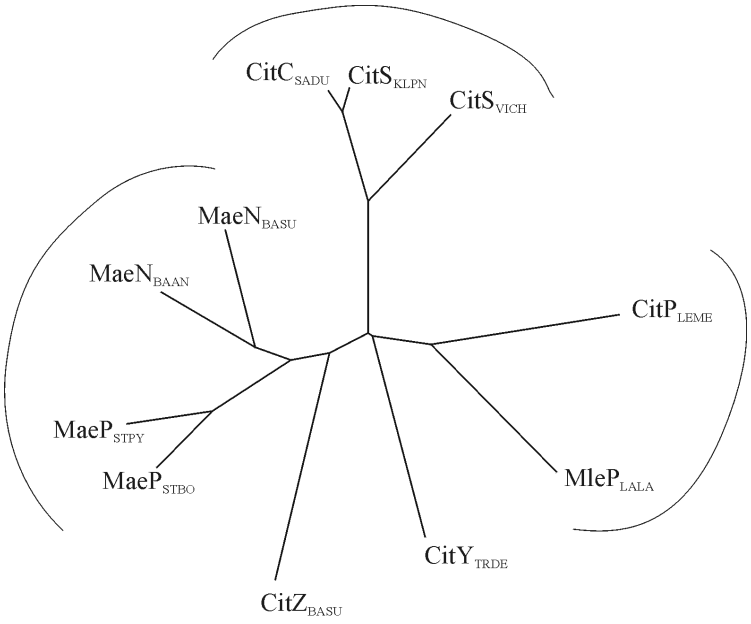

Figure 2. **Phylogenetic tree for the members of the 2-HCT transporter family.** The tree is based on the multiple alignment of 2-HCT transporters with less than 95 % sequence identity (see Table 1). The DRAWTREE program from the PHYLIP package (49) was used for its construction.

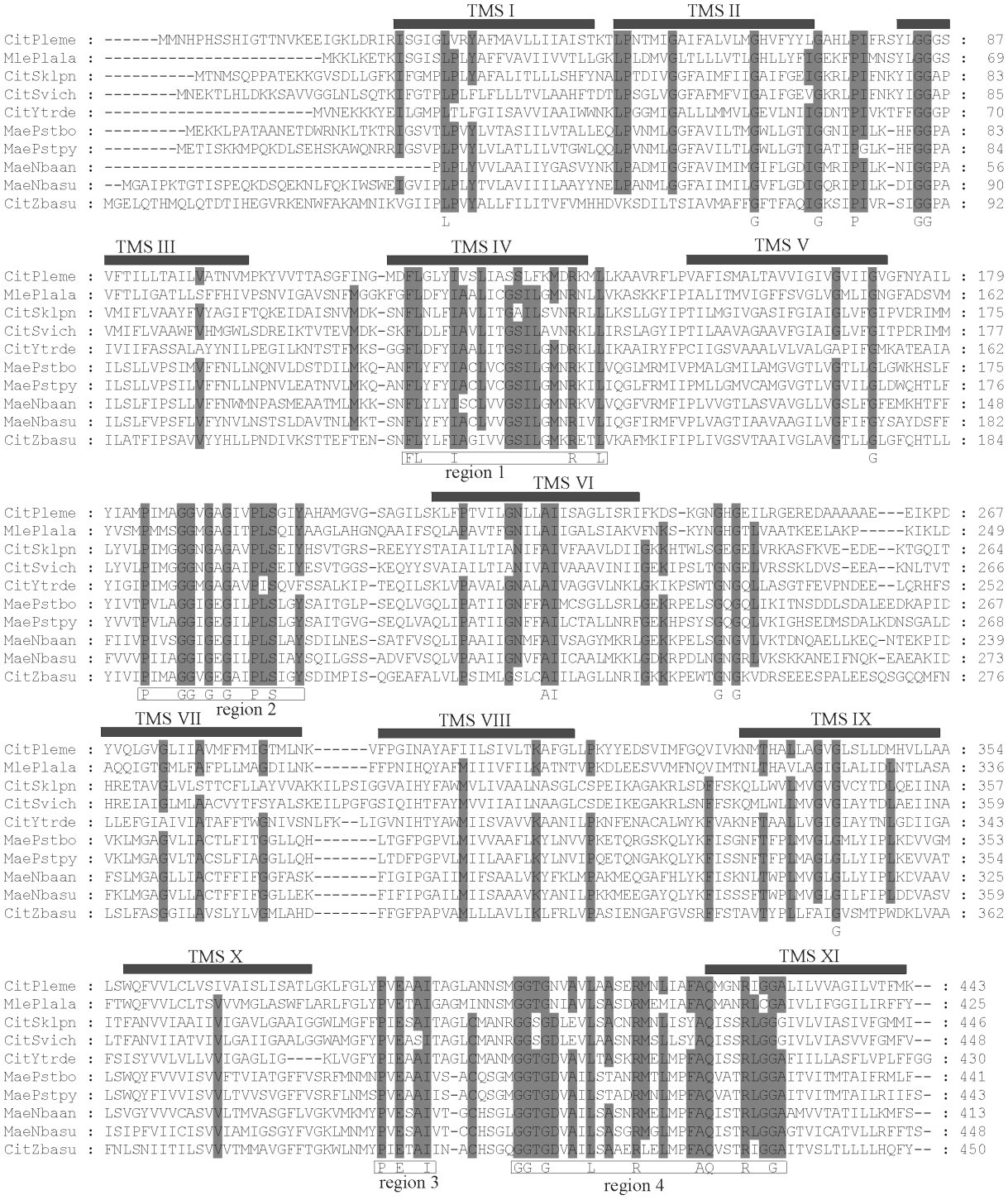

Figure 3. **Multiple sequence alignment of the 2-HCT members.** The alignment was made with Clustal X (11) using only sequences with less than 80% sequence identity. Fully conserved residues are indicated below the alignment. Boxes around these residues indicate conserved regions. Residues that are conserved in 8 out of 10 sequences are indicated by a gray background. Black bars indicate putative transmembrane segments based on topology studies of $CitS_{KLPN}$ (24-27). Abbreviated names of transporter are as in Table 1. The preliminary sequences of $MaeN_{BAAN}$, $MaeP_{STPY}$, and $CitY_{TRDE}$ were obtained from The Institute for Genomic Research website http://www.tigr.org. The preliminary sequence of $MaeN_{BAAN}$ lacks a small part at the N-terminus that was not yet sequenced.

A multiple sequence alignment of the 2-HCT family proteins with less than 80% identical residues is shown in Figure 3. Putative transmembrane segments (TMS) according to the topology model for $CitS_{KLPN}$ (see below) are indicated. The sequence alignment of the 2-HCT proteins reveals 4 conserved regions. Region 4, located partly in putative TMS XI, is suggested to be involved in substrate binding and specificity (18). In $CitP_{LEME}$, the conserved Arg425, located in this region complexes one of the two charged carboxylates in (S)-malate and citrate (see below) (19). Another conserved region that resides in a putative TMS is region 1. This region contains a conserved Arg residue that is a good candidate for interacting with the other charged carboxylate in (S)-malate and citrate (see below). Region 3 is a small stretch of conserved residues that contains a Glu residue which is the only conserved negative charge in the family. In the lactose permease from *E. coli* a Glu residue was shown to be involved in the interaction with the cotransported proton (20). Moreover, Glu residues were found to take part in essential charge pair interactions that are involved in substrate binding and conformational changes (20,21). Similar interactions can be speculated for the 2-HCT proteins. Region 2 consist of a large number of conserved Gly and Pro residues located in the putative loop between TMSs V and VI.

SECONDARY STRUCTURE AND MEMBRANE TOPOLOGY

Figure 4 shows the average hydropathy profile derived from the multiple sequence alignment shown in Figure 3. The hydropathy profiles of the 2-HCT family members are very well conserved. This supports the notion that the global structure, which includes membrane topology, of the 2-HCT members are well conserved (22,23). Secondary structure predictions on the hydropathy profile of $CitS_{KLPN}$ predicted 12 transmembrane segments (24). The membrane topology of $CitS_{KLPN}$ was extensively studied in the *E. coli* membrane using a combination of alkaline phosphatase (PhoA) fusions, site directed Cys labelling and insertion of reporter peptides such a his tags and biotin acceptor molecules (24-26). Moreover the potency of each individual putative TMS to insert into the endoplasmic reticulum membrane was investigated using an in vitro translation system (27). In contrast with the predicted 12 TMSs, the protein was shown to contain 11 TMSs that traverse the membrane in a zig-zag fashion with the N-terminus on the cytoplasmic and the C-terminus on the extracellular face of the membrane. The model contains 4 loops that are considerably longer than usually observed in bacterial secondary transporters. It was speculated that these loops play a role in the structure and/or function of the 2-HCT transporters (26). The extracellular loop between TMS V and VI is about 40 residues long and contains a strong hydrophobic stretch with a high

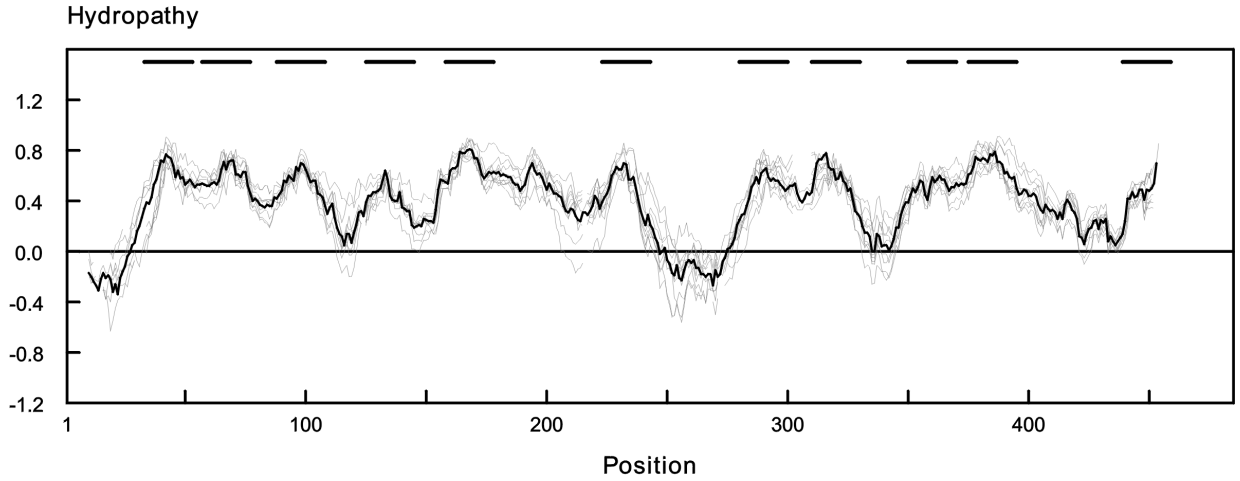

Figure 4. **Average hydropathy profile for the members of the 2-HCT family.** A sliding window of 20 amino acids was used. Black line indicates the average hydropathy profile. Gray lines indicate hydropathy profiles of the individual 2-HCT proteins. Black bars indicate putative transmembrane segments based on topology studies of $CitS_{KLPN}$ (24-27).

degree of sequence conservation (region 2, Figure 3). This provokes the speculation that it can fold between transmembrane segments without completely crossing the membrane, resembling the large reinsertion loop postulated for the glutamate transporters (28). The cytoplasmic loops between TMSs X and XI of about 45 residues was also found to be highly conserved but not hydrophobic. In the primary structure, this loop is in close proximity to Arg 425 that binds the substrate. Limited insertion mutagenesis suggested a defined structure for this region (26). The cytoplasmic loop between TMSs VIII and IX contains no conserved residues but has a strong amphipathic character when folded as an α-helix, which is conserved throughout the 2-HCT family. It was speculated to form a surface helix, exposing its hydrophobic face towards the membrane (26).

TRANSPORT MECHANISM

All characterised 2-HCT proteins were shown to transport citrate or (S)-malate in a unidirectional mode which requires either an electrochemical proton or sodium ion gradient or both to drive transport (Table 1). It should be noted that since citrate and (S)-malate are weak acids, a physiological proton gradient will drive uptake even in the absence of cotransported protons (3). The stoichiometry of substrate and cotransported ions differs among the transporters. The $CitP_{LEME}$ and $MleP_{LALA}$ proteins are thought to transport divalent negative citrate and (S)-malate respectively, in symport with one proton (6,8), whereas reports for $MaeP_{STBO}$ (14) can be interpreted as symport of divalent negative (S)-malate with at least 3 protons. Interestingly, for $CitP_{LEME}$ it was suggested that the lipid environment plays a role in the stoichiometry of the transporter. Citrate transport by $CitP_{LEME}$ expressed in *E. coli* was found to be electroneutral whereas when expressed in *L. lactis* it was electrogenic suggesting a difference in the number of cotransported protons (29). Similarly, the stoichiometry of $CitS_{KLPN}$ is under debate. Expression of $CitS_{KLPN}$ in *E. coli* suggested transport of divalent citrate in symport with two sodium ions and one proton (30-32). However $CitS_{KLPN}$ reconstituted in proteoliposomes suggested the symport of two sodium ions together with the antiport of one sodium and one hydroxyl ion (12,33). These observations suggest that the different stoichiometries may depend on minor conformational changes.

Precursor-product exchange is not fundamentally different from unidirectional transport. In principle, all secondary transporters can catalyze exchange between internal and external substrates as a partial reaction of complete turnover. However, usually this exchange has no physiological relevance since most transporters are specific for one substrate, thus the same molecules are transported in both directions (homologous exchange). Homologous exchange was shown for $CitS_{KLPN}$, $CitP_{LEME}$ and $MleP_{LALA}$ (5). The ability to accept lactate as a substrate allows $CitP_{LEME}$ and $MleP_{LALA}$ to catalyze citrate-lactate or (S)-malate-lactate exchange, respectively (heterologous exchange) (5). In contrast to $CitP_{LEME}$ and $MleP_{LALA}$, $CitS_{KLPN}$ is unable to catalyze precursor-product exchange due to its narrow substrate specificity (5). It does not accept lactate or any of the metabolic endproducts of citrate fermentation in *K. pneumoniae* as a substrate (5,12). Thus the ability to catalyze precursor-product exchange is closely related to a broad specificity. For the other 2-HCT proteins no data is present to conclude or exclude their ability to accept lactate or any other metabolic endproduct of citrate or malate breakdown as a substrate.

Exchange can work according to two different kinetic models, i.e. simultaneous and ping pong mechanisms (34,35). In a ping pong mechanism the substrate binds at one side of the membrane, becomes translocated and is released at the other side after which the reaction proceeds in the reverse direction. In a simultaneous mechanism there are two substrate binding sites, one at each face of the membrane, and substrate must bind simultaneously from two sides before translocation can occur. Only few secondary transporters have been analyzed with respect to their mechanism of exchange. Two examples of bacterial systems, i.e. the arginine-ornithine exchanger from *Lactococcus lactis* (36) and the lactose permease from *E. coli* (37) were found to function according to a ping pong mechanism. Most mitochondrial exchangers,

however, exhibit a simultaneous mechanism (38). The latter are thought to function as homodimers in which the monomers present their respective binding sites to opposite sides of the membrane exchanging the two substrates in a concerted step. Surprisingly, kinetic analysis of homologous exchange by $CitS_{KLPN}$ suggested this transporter to exchange via a simultaneous mechanism which was also explained by postulating a functional homodimer (12). Biochemical evidence in agreement with an oligomeric state of $CitS_{KLPN}$ was presented (39). However, it is not known if such a state is a requirement for activity.

The SUBSTRATE BINDING SITE

The 2-hydroxycarboxylate motif. All characterised 2-HCT transporters were observed to recognise either citrate or (S)-malate as a substrate. In addition, the precursor-product exchangers $CitP_{LEME}$ and $MleP_{LALA}$ recognise (R)- and (S)-lactate as a substrates (5,40). A common motif in these substrates is the 2-hydroxycarboxylate motif, i.e. $HO\text{-}CR_2\text{-}COO^-$ (Figure 1, inset) which led to the term 2-hydroxycarboxylate transporter (2-HCT). The two R groups in this motif were defined as R_R and R_S to discriminate between different positions of the R groups in chiral substrates. Substrate specificity differences between the transporters are due to differences in tolerance towards various R groups. Transporters like $CitS_{KLPN}$ and $CitC_{SADU}$ only tolerate the R groups of citrate while $MaeP_{STBO}$ and $MaeN_{BASU}$ seem specific for the R groups of (S)-malate. $CitP_{LEME}$ and $MleP_{LALA}$ have a low specificity for the R groups. Next to their physiological substrates they transport a wide range of 2-hydroxycarboxylates with R groups ranging from hydrogen atoms to a phenyl group (40). The broad specificity towards the R groups allows $CitP_{LEME}$ and $MleP_{LALA}$ to accept both divalent substrates (citrate or (S)-malate) and monovalent substrates ((R)- and (S)-lactate) and thus function as precursor-product exchangers (5).

Interactions with the R groups of the 2-hydroxycarboxylate motif. The broad specificity, of $MleP_{LALA}$ and $CitP_{LEME}$, towards the R groups was used to study the effect that various R groups have on affinity and translocation efficiency. Comparing affinities between various substrates yielded the postulation of two types of interactions at the R groups (40). Firstly, a strong, possibly electrostatic, interaction was postulated to take place between the protein and the charged carboxylate at the R_S group present in divalent substrates as citrate or (S)-malate. This would explain the high affinity for these substrates as compared to the lower affinity for monovalent substrates such as lactate, that lack this "second" carboxylate group. Secondly, a less strong, possibly hydrophobic, interaction was postulated to explain the higher affinity for a number of substrates with various hydrophobic R_R or R_S groups when compared to similar substrates that have a hydrogen atom instead of a hydrophobic group (40). Both types of interactions were postulated for both $MleP_{LALA}$ and $CitP_{LEME}$ indicating that the architecture of the substrate binding site is quite similar. Differences in substrate specificity between $CitP_{LEME}$ and $MleP_{LALA}$ are caused by subtle differences in the affinity and translocation efficiency and are (i) $MleP_{LALA}$ binds but does not translocate substrates with large R_R groups while $CitP_{LEME}$ both binds and translocates such compounds (ii) $MleP_{LALA}$ has a low affinity for citrate (K_i = 9 mM) as compared to $CitP_{LEME}$ (K_i = 0.056 mM) and is unable to translocate this compound due to its large R_R group, (iii) $CitP_{LEME}$ has a low affinity for substrates with bulky R_S groups in contrast to $MleP_{LALA}$, (iiii) affinity for divalent substrates is lower in $MleP_{LALA}$ than in $CitP_{LEME}$ while affinity for most monovalent substrates is higher (18,40).

The differences in substrate specificity between $MleP_{LALA}$ and $CitP_{LEME}$ were exploited to identify domains that may be part of the substrate binding site and the translocation pore. Chimeras were constructed in and it was shown that the a stretch of 46 residues at the C-terminus, containing conserved region 4, is involved in the binding of hydrophobic R_R and R_S groups of monovalent substrates (18). Interestingly, not all interactions with the hydrophobic R groups were found to be located in the C-terminus. The relative affinity changes upon interchanging the C-terminus differed depending on the structure of the hydrophobic R group

indicating that different R groups interact with different groups on the protein which are not all contained in the C-terminus. Moreover, the C-terminus was thought not to contain residues responsible for the citrate and (S)-malate affinity differences between the transporters. The C-terminal 46 residues encompass the conserved region 4 and putative TMS XI.

Site directed mutagenesis of $CitP_{LEME}$ showed the conserved Arg425, located in putative TMS XI, to interact specifically with the "second" carboxylate, present in divalent substrates but not in monovalent substrates (19). Removal of the positive charge on the protein by an Arg425Cys substitution resulted in a decrease in affinity for the divalent (S)-malate but not for the monovalent 2-hydroxyisobutyrate. Moreover, chemical modification of residues at position 425 was blocked by the presence of substrate. Although other positively charged groups at position 425 can in part take over the function of the guanidinium group only a guanidinium group was able to result in a high affinity interaction with the substrate. It was suggested that the guanidinium group forms two hydrogen bridges with the carboxylate. The ion pair nature of these hydrogen bonds would mean that they are an order of magnitude stronger than those between neutral moieties. Alternatively (or in addition), the guanidinium could make hydrogen bonds with surrounding residues which allows it to position its charge more accurately than other positive groups. Analogous to the 2-HCT transporters, arginine residues have been shown to be involved in the binding of citrate with the citrate-malate exchanger from the mitochondrial inner membrane (17) and with several soluble proteins (41-43). The topology model places Arg425 in TMS XI; hence TMS XI could be lining the translocation pathway. Interestingly, the current topology model places Arg425 close to the cytoplasmic face of the membrane. This view is supported by accessibility studies in membrane vesicles using membrane permeable and impermeable chemical reagents (19).

Interactions with the carboxylate and hydroxyl groups of the 2-hydroxycarboxylate motif. The conserved specificity of the 2-HCT proteins for 2-hydroxycarboxylic substrates suggests an important role for the carboxylate and hydroxyl groups of the motif in substrate recognition. This was experimentally verified using substrate analogs, which were modified at one of the respective groups (40). Substrate analogs in which the carboxylate group was methylated or replaced by a hydroxyl or an aldehyde group were not bound by $CitP_{LEME}$ or $MleP_{LALA}$. It was speculated that the negatively charged carboxylate forms an absolutely essential interaction with a, most likely positively charged, residue on the protein. The strongly conserved specificity, of the 2-HCT transporters, for 2-hydroxycarboxylates suggests the involvement of strongly conserved residues in the interaction. In addition to Arg425, two conserved positive charges are present in the 2-HCT family, i.e. Arg138 and Arg413. These residues are good candidates to interact with the carboxylate of the 2-hydroxycarboxylate motif in a similar fashion as the interaction between Arg425 and the carboxylate at the R_S position of divalent substrates. An Arg413Cys substitution in $CitP_{LEME}$ had no effect on the affinity for (S)-malate (M. Bandell, unpublished results) leaving Arg138 ($CitP_{LEME}$ numbering) to be the most likely residue. Arg138 is located in putative TMS IV which is highly conserved (conserved region 1). Like Arg425, the topology model predicts Arg138 close to the cytoplasmic face of the membrane. This would allow a close proximity of these residues if TMSs IV and XI are next to each other in the helix packing.

Substrate analogs that were modified at the hydroxyl group of the 2-hydroxycarboxylate motif exhibited drastically reduced affinities. Substrate analogs in which the hydroxyl of the motif was replaced by a hydrogen atom were not bound by $CitP_{LEME}$, $MleP_{LALA}$, $CitS_{KLPN}$ or $MaeP_{STBO}$ (5,14,40). However, $CitP_{LEME}$ was able to transport and bind to a low extent 3-hydroxycarboxylic compounds in which the distance between the hydroxyl and the carboxylate of the motif is increased with one C-atom (5,40). Moreover, transport of substrate analogs in which the hydroxyl was replaced with an oxo group, i.e.oxaloacetate and pyruvate, was observed for $CitP_{LEME}$ and $MleP_{LALA}$ albeit with a drastic loss of affinity (5,40). The hydroxyl group was speculated to form a hydrogen bond with a residue on the protein that can acts as both a hydrogen acceptor as well as a hydrogen donor. This way both an oxo and a hydroxyl

group can be bound. Flexibility of the substrate molecule and/or protein may account for the activity with 3-hydroxycarboxylates. Similar to the interaction with the carboxylate of the motif, the interaction with the hydroxyl of the motif is though to be well conserved throughout the 2-HCT family. There are six conserved residues that would be able to form a hydrogen bond with the hydroxyl group. These are Arg138, Ser198, Glu387, Gln421, Arg413 and Arg425 ($CitP_{LEME}$ numbering). The latter two residues are shown to be not involved in the interaction with the hydroxyl since mutagenesis of these residues did not lead to a dramatic decrease in affinity for all substrates, as would be expected ((19), M. Bandell unpublished results). Of the remaining four residues Gln421 seems the best candidate since it is located in TMS XI exactly one α-helical turn towards the cytoplasmic side of Arg425.

Model of the binding site of $CitP_{LEME}$. A combination of substrate specificity studies, topology studies, chimeras and site directed mutants allowed us to form a model of the substrate binding site (Figure 5). In the model the conserved C-terminal part of the transporters, containing putative TMS XI and part of the preceding loop structure, plays an important role in substrate affinity. TMS XI contains Arg425 which interacts with a carboxylate at the R_S position of divalent substrates. The interaction is though to be a combination of an electrostatic interaction together with the formation of two H bridges. The C-terminus also contains residues that promote affinity for hydrophobic R_R and R_S groups. Most likely these residues are located in close proximity of Arg425 in TMS XI. Interestingly, the protein interacts with the hydrophobic R groups via an array of interactions, not all of which are located in the C-terminus. Especially the interactions with the protonated carboxylate at the R_R group of citrate are located outside the C-terminus. The interaction between the protein and the carboxylate of the 2-hydroxycarboxylate motif is not yet identified. However, based on the combined structure-function data presented here, we propose Arg 138, located in TMS IV, to be the most likely candidate to bind the carboxylate. Furthermore, we suggest the most likely candidate to interact with the hydroxyl is suggested to be Gln421, located in TMS XI, just one helical turn towards the cytoplasmic side of Arg 425. Arg425 seems to reside close to the cytoplasmic face of the membrane. This poses the question of how hydrophilic substrates reach a substrate binding site that is buried in the hydrophobic environment of the membrane. Possibly TMS XI (in combination with TMS IV) forms a hydrophilic cleft leading towards the binding site.

The ability to translocate substrates that differ in structure (and charge) is a property of only few transporters. Most well known are the mitochondrial exchangers, the bacterial precursor-product exchangers and the multi drug transporters. The mechanisms behind their promiscuity may be similar. Studies on the multi drug transporters MdfA (44) and QacA (45) identified negative charges in transmembrane segments that interact specifically with positively charged substrates. Moreover, multi drug transporters are thought to interact with different neutral

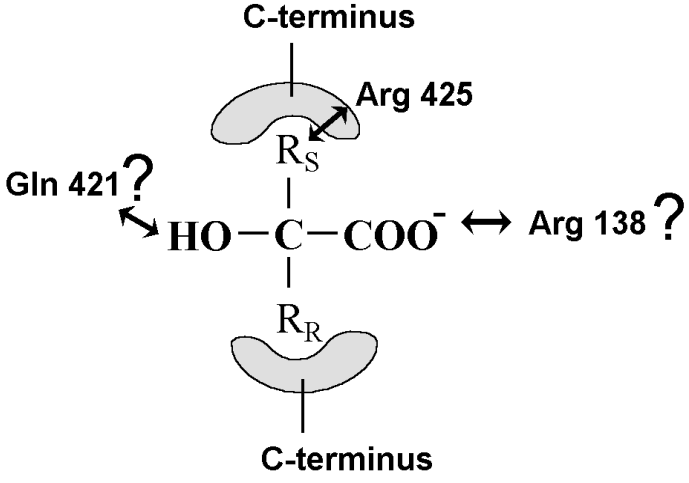

Figure 5. **Model of the binding site of $CitP_{LEME}$.** The 2-hydroxycarboxylate motif and its interaction sites. Arg425 interacts with charged carboxylates at the R_S group. Residues in the C-terminal 46 residues (gray patches indicated as C-terminus) interact with neutral R groups. Question marks indicate the most likely residues that interact with the substrates (see text).

substrates via different sets of interactions (46-48). These properties are similar to what was described for the interactions between $CitP_{LEME}$ and $MleP_{LALA}$ and the R groups of various substrates.

ACKNOWLEDGMENTS

We thank Bastiaan Krom for his data on the CitZ protein of *B. subtilis*. Preliminary sequence data was obtained from The Institute for Genomic Research and the National Centre for Biotechnology Information Sequencing of *B. anthracis* and *T. denticola* was accomplished in cooperation with the University of Texas and with support from ONR, DOE and NIAID organisations. Sequencing of *S. pyogenis* was accomplished by the University of Oklahoma.

OUTLINE OF THE THESIS

The object of the research described in this thesis was to identify substrate-protein interactions that provide affinity and specificity for $CitP_{LEME}$ from *Lc.mesenteroides* and $MleP_{LALA}$ from *L. lactis*. The ability of these transporters to transport substrates that differ in structure and charge implied specific requirements for the substrate binding site.

Chapter 2 describes the cloning of the *citP* gene from *Lc. mesenteroides* and the subsequent expression and characterisation in a citrate/malate transport deficient *L. lactis* strain and in *E. coli*. The *citP* gene codes for the citrate transporter that is involved in precursor-product exchange between citrate and lactate. This finding resolved a controversy on the characteristics of citrate transport by CitP proteins from different lactic acid bacteria.

Chapter 3 describes the cloning, expression and characterisation of the *mleP* gene from *Lactococcus lactis*. The *mleP* gene codes for the (S)-malate transporter of *L. lactis* that is involved in the precursor-product exchange between malate and lactate. $MleP_{LALA}$ is homologous to $CitP_{LEME}$ and $CitS_{KLPN}$ from respectively *Lc. mesenteroides* and *K. pneumoniae*. Substrate specificities of $MleP_{LALA}$, $CitP_{LEME}$ and $CitS_{KLPN}$ were determined and compared. All three proteins were found to be specific for substrates containing a 2-hydroxycarboxylate motif, i.e. $HO\text{-}CR_2\text{-}COO^-$. The precursor-product exchangers $CitP_{LEME}$ and $MleP_{LALA}$ were found to have a broad substrate specificity, transporting 2-hydroxycarboxylates with various R groups. In contrast $CitS_{KLPN}$ was found to have a narrow substrate specificity transporting mainly citrate

Chapter 4 elaborates on the substrate specificity studies of chapter 3 and describes the kinetic characterisation of $CitP_{LEME}$ and $MleP_{LALA}$ with a number of substrates and substrate analogs. This suggested that the interactions between the protein and the OH and COO^- of the 2-hydroxycarboxylate motif are essential for affinity. Moreover, hydrophobic and electrostatic interactions with the R groups are postulated to be not essential but modulate affinity and translocation efficiency. Substrate specificity differences between $MleP_{LALA}$ and $CitP_{LEME}$ are due to subtle differences in either K_m or V_{max}. $MleP_{LALA}$ has in most cases a higher affinity for monovalent substrates and a lower affinity for divalent substrates than $CitP_{LEME}$. Moreover citrate is no substrate of $MleP_{LALA}$ due to a very low V_{max} for this substrate.

Chapter 5 describes the construction of chimeras between $CitP_{LEME}$ and $MleP_{LALA}$. This revealed that the C-terminal 46 residues is involved in the interactions of the transporters with uncharged R groups of monovalent substrates.

Chapter 6 elaborates on the results of chapter 5. Results are described which indicate that Arg425 of $CitP_{LEME}$, present in the C-terminal region, identified in chapter 5, interacts with the charged carboxylate present in divalent but not in monovalent substrates. Accessibility studies using membrane permeable and impermeable chemical modification agents suggest that this residue is located at the cytoplasmic face of TMS XI.

REFERENCES

1. Saier, M. H. (2000) *Microbiol.Mol.Biol.Rev.* **64,** 354-411
2. Lolkema, J. S., Poolman, B., and Konings, W. N. (1996) In Konings, W. N., Kaback, H. R., and Lolkema, J. S., editors. *Handbook of Biological Physics*, Elsevier, Amsterdam

3. Konings, W. N., Lolkema, J. S., and Poolman, B. (1995) *Arch.Microbiol.* **164,** 235-242
4. Palmieri, F., Bisaccia, F., Capobianco, L., Dolce, V., Fiermonte, G., Iacobazzi, V., Indiveri, C., and Palmieri, L. (1996) *Biochim.Biophys.Acta* **1275,** 127-132
5. Bandell, M., Ansanay, V., Rachidi, N., Dequin, S., and Lolkema, J. S. (1997) *J.Biol.Chem.* **272,** 18140-18146
6. Poolman, B., Molenaar, D., Smid, E. J., Ubbink, T., Abee, T., Renault, P. P., and Konings, W. N. (1991) *J.Bacteriol.* **173,** 6030-6037
7. Marty Teysset, C., Posthuma, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1996) *J.Bacteriol.* **178,** 2178-2185
8. Marty Teysset, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1995) *J.Biol.Chem.* **270,** 25370-25376
9. Magni, C., de Mendoza, D., Konings, W. N., and Lolkema, J. S. (1999) *J.Bacteriol.* **181,** 1451-1457
10. Garcia Quintans, N., Magni, C., de Mendoza, D., and Lopez, P. (1998) *Appl.Environ.Microbiol.* **64,** 850-857
11. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389-3402
12. Pos, K. M. and Dimroth, P. (1996) *Biochemistry* **35,** 1018-1026
13. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) *Nucleic Acids Res.* **25,** 4876-4882
14. Kawai, S., Suzuki, H., Yamamoto, K., and Kumagai, H. (1997) *J.Bacteriol.* **179,** 4056-4060
15. Wei, Y., Guffanti, A. A., Ito, M., and Krulwich, T. A. (2000) *J.Biol.Chem.* in press
16. Ishiguro, N., Izawa, H., Shinagawa, M., Shimamoto, T., and Tsuchiya, T. (1992) *J.Biol.Chem.* **267,** 9559-9564
17. Pao, S. S., Paulsen, I. T., and Saier, M. H., Jr. (1998) *Microbiol.Mol.Biol.Rev.* **62,** 1-34
18. Bandell, M. and Lolkema, J. S. (2000) *Biochemistry* in press
19. Bandell, M. and Lolkema, J. S. (2000) *J.Biol.Chem.* in press
20. Kaback, H. R. (1997) *Proc.Natl.Acad.Sci.U.S.A* **94,** 5539-5543
21. Venkatesan, P. and Kaback, H. R. (1998) *Proc.Natl.Acad.Sci.U.S.A* **95,** 9802-9807
22. Lolkema, J. S. and Slotboom, D. J. (1998) *Mol.Membr.Biol.* **15,** 33-42
23. Lolkema, J. S. and Slotboom, D. J. (1998) *FEMS Microbiol.Rev.* **22,** 305-322
24. van Geest, M. and Lolkema, J. S. (1996) *J.Biol.Chem.* **271,** 25582-25589
25. van Geest, M. and Lolkema, J. S. (1999) *J.Biol.Chem.* **274,** 29705-29711
26. van Geest, M. and Lolkema, J. S. (2000) *Biochim.Biophys.Acta* **1466,** 328-338
27. van Geest, M., Nilsson, I., von Heijne, G., and Lolkema, J. S. (1999) *J.Biol.Chem.* **274,** 2816-2823
28. Slotboom, D. J., Sobczak, I., Konings, W. N., and Lolkema, J. S. (1999) *Proc.Natl.Acad.Sci.U.S.A* **96,** 14282-14287
29. Bandell, M., Lhotte, M. E., Marty Teysset, C., Veyrat, A., Prevost, H., Dartois, V., Divies, C., Konings, W. N., and Lolkema, J. S. (1998) *Appl.Environ.Microbiol.* **64,** 1594-1600
30. van der Rest, M. E., Molenaar, D., and Konings, W. N. (1992) *J.Bacteriol.* **174,** 4893-4898
31. van der Rest, M. E., Siewe, R. M., Abee, T., Schwarz, E., Oesterhelt, D., and Konings, W. N. (1992) *J.Biol.Chem.* **267,** 8971-8976
32. Lolkema, J. S., Enequist, H., and van der Rest, M. E. (1994) *Eur.J.Biochem.* **220,** 469-475
33. Dimroth, P. and Thomer, A. (1990) *J.Biol.Chem.* **265,** 7721-7724
34. Fromm, H. J. (1979) *Methods Enzymol.* **63,** 42-53
35. Rudolph, F. B. and Fromm, H. J. (1979) *Methods Enzymol.* **63,** 138-159
36. Driessen, A. J. M., Molenaar, D., and Konings, W. N. (1989) *J.Biol.Chem.* **264,** 10361-10370
37. Garcia, M. L., Viitanen, P., Foster, D. L., and Kaback, H. R. (1983) *Biochemistry* **22,** 2524-2531
38. Palmieri, F. (1994) *FEBS Lett.* **346,** 48-54
39. Pos, K. M., Bott, M., and Dimroth, P. (1994) *FEBS Lett.* **347,** 37-41
40. Bandell, M. and Lolkema, J. S. (1999) *Biochemistry* **38,** 10352-10360
41. Karpusas, M., Branchaud, B., and Remington, S. J. (1990) *Biochemistry* **29,** 2213-2219
42. Wilks, H. M., Hart, K. W., Feeney, R., Dunn, C. R., Muirhead, H., Chia, W. N., Barstow, D. A., Atkinson, T., Clarke, A. R., and Holbrook, J. J. (1988) *Science* **242,** 1541-1544
43. Hall, M. D., Levitt, D. G., and Banaszak, L. J. (1992) *J.Mol.Biol.* **226,** 867-882
44. Edgar, R. and Bibi, E. (1999) *EMBO J.* **18,** 822-832
45. Paulsen, I. T., Brown, M. H., Littlejohn, T. G., Mitchell, B. A., and Skurray, R. A. (1996) *Proc.Natl.Acad.Sci.U.S.A* **93,** 3630-3635
46. Zheleznova, E. E., Markham, I., Edgar, I., Bibi, I., Neyfakh, A. A., and Brennan, R. G. (2000) *Trends Biochem.Sci.* **25,** 39-43
47. Zheleznova, E. E., Markham, P. N., Neyfakh, A. A., and Brennan, R. G. (1999) *Cell* **96,** 353-362
48. Vazquez-Laslop, N., Markham, P. N., and Neyfakh, A. A. (1999) *Biochemistry* **38,** 16925-16931
49. Felsenstein J. (1993) PHYLIP (Phylogeny Inferrence Package). Version 3.5c. Department of Genetics, University of Washington, Seattle, Distributed by the author.
50. Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Umayam, L., Gill, S. R., Nelson, K. E., Read, T. D., Tettelin, H., Richardson,

D., Ermolaeva, M. D., Vamathevan, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R. D., Nierman, W. C., White, O., Salzberg, S. L., Smith, H. O., Colwell, R. R., Mekalanos, J. J., Venter, J. C., and Fraser, C. M. (2000) *Nature* **406,** 477-483

51. Kunst, F., Ogasawara, N., Moszer, I., and 148 other authors (1997) *Nature* **390,** 249-256

Chapter 2

Mechanism of the Citrate Transporters (CitPs) in Carbohydrate and Citrate Cometabolism in *Lactococcus* and *Leuconostoc* Species

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SUMMARY

Citrate metabolism in the lactic acid bacterium *Leuconostoc mesenteroides* generates an electrochemical proton gradient across the membrane by a secondary mechanism (27). Reports on the energetics of citrate metabolism in the related organism *Lactococcus lactis* are contradictory and this study was performed to clarifying this issue. Cloning of the membrane potential generating citrate transporter (CitP) of *Leuconostoc mesenteroides* revealed an amino acid sequence that is almost identical to the known sequence of the CitP of *Lactococcus lactis.* The cloned gene was expressed in a *Lactococcus lactis* Cit$^-$ strain, and the gene product was functionally characterized in membrane vesicles. Uptake of citrate was counteracted by the membrane potential, and the transporter efficiently catalyzed heterologous citrate-lactate exchange. These properties are essential for the generation of a membrane potential under physiological conditions and show that the *Leuconostoc* CitP retains its properties when embedded in the cytoplasmic membrane of *Lactococcus lactis*. Furthermore, using the same criteria and experimental approach, we demonstrated that the endogenous citrate transporter CitP of *Lactococcus lactis* has the same properties, showing that the few differences in the amino acid sequences of the CitPs of members of the two genera do not result in a different catalytic mechanisms. The results strongly suggest that the energetics of citrate degradation in *Lactococcus lactis* and *Leuconostoc mesenteroides* are the same; i.e., citrate metabolism in *Lactococcus lactis* is a proton motive force generating pathway.

INTRODUCTION

Only a few strains of lactic acid bacteria are able to ferment citrate. The ability to metabolize citrate is invariably linked to endogenous plasmids that contain the gene encoding the transporter that is responsible for the uptake of citrate from the medium. The citrate transporters (CitPs) have been found in strains belonging to the genera of the *Lactococcus* and *Leuconostoc.* In the dairy industry, these *Lactococcus* and *Leuconostoc* stains are used in mixed cultures, and the metabolism of citrate is important in many fermentation processes because of the formation of carbon dioxide, diacetyl, acetoin and butanediol, compounds that contribute to the organoleptic properties of the fermentation products (for a review see reference 11).

In recent studies we have described in detail the energetics of citrate metabolism in *Leuconostoc mesenteroides* (26, 27). Citrate fermentation results in the formation of an electrochemical proton gradient across the cell membrane (proton motive force) by a secondary mechanism (16, 21) in which CitP plays a crucial role. The transporter catalyzes exchange of divalent anionic citrate and monovalent lactate, which results in the generation of a membrane potential with physiological polarity (i.e., the inside is negative) (Figure 1). Lactate is a product

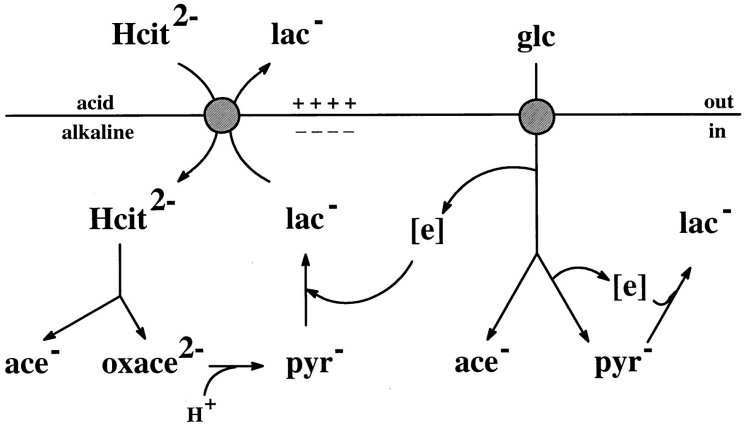

Figure 1. **Schematic of the mechanism of proton motive force generation by citrolactic fermentation in *Lc. mesenteroides*.** Citrate metabolism and glucose metabolism are shown on the left and right, respectively. Electrogenic exchange of citrate and lactate by CitP results in the generation of a membrane potential. Scalar proton consumption in the decarboxylation of oxaloacetate results in the generation of a transmembrane pH gradient. During cometabolism, redox equivalents are shunted from glucose metabolism to citrate metabolism. Hcit, citrate; lac, lactate; ace, acetate; oxace, oxaloacetate; pyr, pyruvate; glc, glucose.

of citrate fermentation during cometabolism with a carbohydrate (citrolactic fermentation). Upon entering the cell, citrate is cleaved by citrate lyase, which yields acetate and oxaloacetate. Decarboxylation of the latter compounds yields carbon dioxide and pyruvate. Pyruvate functions as a sink for the reducing equivalents produced in the heterofermentative carbohydrate degradation pathway and is converted to the end product lactate (3) which leaves the cell in exchange for citrate (precursor-product exchange). The decarboxylation of oxaloacetate plays an additional role in the metabolic energy generation since it consumes a cytoplasmic proton, which results in the generation of a transmembrane pH gradient of physiological polarity (i.e., the inside is alkaline). Together, the charge translocation catalyzed by the transporter and the proton consumption in the decarboxylation step are equivalent to the pumping of a proton out of the cell (20, 21).

In citrate fermenting *Lactococcus* species the initial steps of citrate breakdown proceed through the same intermediates as those observed in *Leuconostoc* species, and the homofermentative carbohydrate metabolism in lactococci is likely to produce enough lactate for the CitP to function as a citrate-lactate exchanger (35). Moreover, the genes coding for the citrate carriers of the *Lactococcus lactis* (*citPll*) and the *Leuconostoc lactis* (*citPlcl*) have been cloned and sequenced and have been found to be almost identical (4, 41). All of this strongly suggests that the energetics of the citrate metabolic pathway described above for *Lc. mesenteroides* is also valid for lactococci. However, the reports on the energy coupling mechanism of the *L. lactis* transporter are contradictory. Uptake studies performed with membrane vesicles prepared from *Escherichia coli* expressing the transporter of *L. lactis* indicated that citrate transport was driven by the proton motive force. Consequently, it was concluded that uptake of citrate in *L. lactis* costs metabolic energy (4). In contrast, measurement of energetic parameters in whole cells of *L. lactis* indicated that metabolic energy was conserved during citrate metabolism possibly via electrogenic exchange of divalent citrate with monovalent acetate or pyruvate (11). The results of yet another study, which involved citrate uptake measurements in whole cells of *L. lactis* again seemed to indicate proton motive force driven uptake of citrate occurs (25).

Different energetics of the citrate degradation pathways in *L. lactis* and *Lc. mesenteroides* would mean that the energy coupling mechanisms of the CitPs must be different. The transporter of *Lc. mesenteroides* has not been cloned and sequenced and could be a different protein. Alternately, small differences in the amino acid sequence of the transporters could be responsible for a different mechanisms or the different lipid environment in the two types of lactic acid bacteria could change the energy coupling mechanism as was recently described for the glutamate transporter of the thermophilic bacterium *Bacillus stearothermophilus* (38). The present study demonstrates that the energetics of the CitPs of *Lactococcus* and *Leuconostoc*

species are the same. The conclusion strongly suggest that citrate metabolism in *L. lactis* results in the generation of a proton motive force.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. *Leuconostoc mesenteroides* subsp. *mesenteroides* 19D was obtained from the collection of the Institute National de la Recherche Agronomique (Jouy en Josas, France) and was grown at 30 °C in MRS medium supplemented with 0.5% glucose with or without 10 mM citrate (5). *Lactococcus lactis* subsp. *lactis* biovar. diacetylactis NCDO176, which was obtained from the Dutch Institute of Dairy Research (Ede, The Netherlands), and *Lactococcus lactis* MG1363 (9) harbouring plasmid pMBcitP were grown at 30 °C in M17 medium supplemented with 0.5% lactose and with or without 10 mM citrate. Plasmid pMBcitP is an *E. coli-L. lactis* shuttle vector that contains the *citP* gene of *Lc. mesenteroides* under control of the *citP* promotor of *L. lactis* and has been described in more detail elsewhere (1). *E. coli* strains JM101 and JM109(DE3) (32) were routinely grown in LB medium at 37 °C under vigourous shaking and, when appropriate, with carbenicilin or ampicillin at a concentration of 50 μg/ml. Citrate utilization by *Lc. mesenteroides* was detected by using the medium of Kempler and McKay (14). Citrate utilization by *E.coli* was examined using Simmons agar (33) and Christensen agar (2).

Genetic manipulations. Unless otherwise indicated, all genetic engineering was performed by standard procedures (32). Endogenous plasmids of *Lc. mesenteroides* were isolated on a small scale as described previously (30) and on a large scale using the alkaline lysis, with the following modifications. Harvested cells were washed twice in TES buffer (30 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, pH 8.0) (18), and the CsCl-ethidium bromide gradient was replaced by an ethanol precipitation (17). *L. lactis* MG1363 was transformed by electroporation (10) and the *E. coli* strains either by electroporation or by the standard $CaCl_2$ treatment procedure. Southern blot hybridizations (34) were performed by using a VacuGene XL vacuum blotting system (Pharmacia) and a 0.45-μm pore size nylon membrane filter (Pharmacia). Labelling of DNA probes, hybridization, and detection were performed using the DIG Labelling and Detection Kit protocols (Boehringer, Mannheim, Germany). DNA hybridization and stringent washes in 150 mM NaCl-15 mM sodium citrate were carried out at 68 °C.

Membrane preparations. Cells of *L. lactis* MG1363 harbouring plasmid pMBcitP and *L. lactis* NCDO176 were harvested at an optical density at 660 nm (OD_{660}) of 1, washed once and resuspended in 50 mM potassium phosphate (pH 7.0) at an OD_{660} of 500, and then rapidly frozen in liquid nitrogen until they were used. Right-side-out membrane vesicles were prepared by the osmotic shock lysis procedure essentially as described previously (31). Membrane vesicles were rapidly frozen and stored in liquid nitrogen until they were used. The protein concentration was determined by the method of Lowry et al. (23). The membranes were fused to proteoliposomes containing beef heart cytochrome *c* oxidase or to liposomes essentially as described (6). L-α-phosphatidyl ethanolamine was purified from 1 g of *E. coli* extract (Avanti polar lipids) by successive washing with acetone and diethyl ether, after which the concentration was determined (7). Cytochrome *c* oxidase isolated from beef heart mitochondria was reconstituted into liposomes by detergent dialysis and reconstituted in a mixture containing the purified *E. coli* lipids and egg phosphatidylcholine at a ratio 3:1. Proteoliposomes containing cytochrome *c* oxidase were fused with the membrane vesicles of *L. lactis* at a ratio of 1 mg of protein per 10 mg of lipids by freezing in liquid nitrogen and then slowly thawing at room temperature (6). The resulting hybrid membranes were made unilamelar by sonication (eight cycles; 15 s on and 45 s off; amplitude 4 to 6 μm) or by extrusion using successively 400- and 200-nm-pore-size polycarbonate filters (29). Right-side-out membrane vesicles of *E. coli* strain JM101 were prepared by the osmotic lysis procedure (13).

Transport assays. *(i) Proton motive force driven uptake in vesicles.* The experiments to determine proton motive force uptake in vesicles were performed in 50 mM potassium phosphate (pH 6) under a flow of water-saturated air at 30 ^{o}C. The membranes were incubated for 10 min in the presence or in the absence of valinomycin and nigericin at concentrations of 1 and 0.5 μM, respectively. Membrane vesicles of *L. lactis* fused to proteoliposomes containing cytochrome *c* oxidase were energized by adding 200 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD), 20 μM cytochrome *c* (from horse heart; Sigma), and 10 mM potassium ascorbate. Membrane vesicles of *E. coli* were energized by adding 200 μM phenazine methosulphate (PMS) and 10 mM potassium ascorbate. After incubation for 1 min, [1,5-^{14}C]citrate was added at a concentration of 4.5 μM. Samples (100 μL) were removed at different times, placed in 2 mL ice-cold 0.1 mM LiCl to stop the reaction, and filtered through 0.45-μm-pore-size cellulose nitrate filters (Schleider & Schull GmbH). Filters were rinsed with 2 mL ice-cold 0.1 mM LiCl and transferred to scintillation vials.

(ii) Exchange measurements. Hybrid membranes obtained by fusion of membrane vesicles of *L. lactis* with liposomes lacking cytochrome *c* oxidase were concentrated by centrifugation (250,000 x *g* for 45 min, °4 C) and incubated with 5 mM [1,5-^{14}C]citrate in 50 mM potassium phosphate (pH 6) for 30 min at 20 °C. After incubation with valinomycin and nigericin, the hybrid membranes were diluted 100-fold with the same buffer with or without 5 mM unlabeled substrates. The samples were processed as described above.

Nucleotide sequence accession number. The nucleotide sequence of the insert in pNA1008 determined in this study has been deposited in the GeneBank database under accession no. L29572

```
                                                  x
CitPlcm      1 MMNHPHSSHIGTTNVKEEIGKLDRIRISGIGLVAYAFMAVLLIIAISTKTLPNTMIGAIF 60
CitPlcl      1 MMNHPHSSHIGTTNVKEEIGKLDRIRISGIGLVAYAFMAVLLIIAISTKTLPNTMIGAIF 60
CitPll       1 MMNHPHSSHIGTTNVKEEIGKLDRIRISGIGLIAYAFMAVLLIIAISTKTLPNTMIGAIF 60

                                                        x
CitPlcm     61 ALVLMGHVFYYLGAHLPIFRSYLGGGSVFTILLTAILVATNVMPKYVVTTASGFINGMDF 120
CitPlcl     61 ALVLMGHVFYYLGAHLPIFRSYLGGGSVFTILLTAILVATNVMPKYVVTTASGFINGMDF 120
CitPll      61 ALVLMGHVFYYLGAHLPIFRSYLGGGSVFTILLTAILVATNVIPKYVVTTASGFINGMDF 120


CitPlcm    121 LGLYIVSLIASSLFKMDRKMLLKAAVRFLPVAFISMALTAVVIGIVGVIIGVGFNYAILY 180
CitPlcl    121 LGLYIVSLIASSLFKMDRKMLLKAAVRFLPVAFISMALTAVVIGIVGVIIGVGFNYAILY 180
CitPll     121 LGLYIVSLIASSLFKMDRKMLLKAAVRFLPVAFISMALTAVVIGIVGVIIGVGFNYAILY 180


CitPlcm    181 IAMPIMAGGVGAGIVPLSGIYAHAMGVGSAGILSKLFPTVILGNLLAIISAGLISRIFKD 240
CitPlcl    181 IAMPIMAGGVGAGIVPLSGIYAHAMGVGSAGILSKLFPTVILGNLLAIISAGLISRIFKD 240
CitPll     181 IAMPIMAGGVGAGIVPLSGIYAHAMGVGSAGILSKLFPTVILGNLLAIISAGLISRIFKD 240


                           xx  xx
CitPlcm    241 SKGNGHGEILRGEREDAAAAAEEIKPDYVQLGVGLIIAVMFFMIGTMLNKVFPGINAYAF 300
CitPlcl    241 SKGNGHGEILRGEREDAAA--EEIKPDYVQLGVGLIIAVMFFMIGTMLNKVFPGINAYAF 298
CitPll     241 SKGNGHGEILRGEREKSAA-AEEIKPDYVQLGVGLIIAVMFFMIGTMLNKVFPGINAYAF 299


CitPlcm    301 IILSIVLTKAFGLLPKYYEDSVIMFGQVIVKNMTHALLAGVGLSLLDMHVLLAALSWQFV 360
CitPlcl    299 IILSIVLTKAFGLLPKYYEDSVIMFGQVIVKNMTHALLAGVGLSLLDMHVLLAALSWQFV 358
CitPll     300 IILSIVLTKAFGLLPKYYEDSVIMFGQVIVKNMTHALLAGVGLSLLDMHVLLAALSWQFV 359


CitPlcm    361 VLCLVSIVAISLISATLGKLFGLYPVEAAITAGLANNSMGGTGNVAVLAASERMNLIAFA 420
CitPlcl    359 VLCLVSIVAISLISATLGKLFGLYPVEAAITAGLANNSMGGTGNVAVLAASERMNLIAFA 418
CitPll     360 VLCLVSIVAISLISATLGKLFGLYPVEAAITAGLANNSMGGTGNVAVLAASERMNLIAFA 419


CitPlcm    421 QMGNRIGGALILVVAGILVTFMK 443
CitPlcl    419 QMGNRIGGALILVVAGILVTFMK 441
CitPll     420 QMGNRIGGALILVVAGILVTFMK 442
```

Figure 2. **The amino acid sequences of citrate transporters CitPs of lactic acid bacteria.** Differences between the sequences are indicated by x. CitP sequencs of *Lc. mesenteroides* 19D (CitPlcm), *Lc. lactis* (CitPlcl) and *L. lactis* (CitPll) are shown.

RESULTS

Cloning and sequencing of the *citP* gene of *Lc. mesenteroides* 19D. Cells of *Lc. mesenteroides* 19D Cit^+ produce dark blue colonies on Kempler-McKay indicator plates. The Cit^+ phenotype is easily and spontaneously lost when citrate is omitted from the growth medium, as shown by the appearance of white colonies on the same plates. Prolonged growth of cells with the Cit^- phenotype in medium containing citrate does not result in revertants, which is consistent with the notion that the Cit^+ phenotype is plasmid encoded (18). Total plasmid DNAs from *Lc. mesenteroides* Cit^+ and Cit^- cultures were isolated, and a size analysis revealed that a 22-kb plasmid was not present in the Cit^- derivatives. The *citP* gene of *L. lactis* NCDO176 hybridized strongly and exclusively with the 22-kb plasmid from the *Lc. mesenteroides* Cit^+ cells and not with any of the plasmid DNAs of the Cit^- derivative. No hybridization was observed with chromosomal DNA isolated from either the parental or the Cit^- strain (data not shown). These results strongly suggest that the *citP* gene of *Lc. mesenteroides* 19D is exclusively located on the 22-kb plasmid.

Total plasmid DNA from *Lc. mesenteroides* 19D was partially restricted with *Sau*3A. Fragments ranging in size from 4- to 9-kb were cloned into the compatible *Bam*HI site of plasmid pUC19. About 400 recombinant plasmids were screened by hybridization using the lactococcal *citP* gene as a probe. Strong hybridization was obtained with plasmid pNA1006 that contained a 5.5-kb insert. Further subcloning was achieved by deleting a *Hin*dIII fragment; this resulted in plasmid pNA1008 that contained a 3.6-kb insert. Plasmid pNA1008 was transformed into *E. coli* JM101, and the transformant was grown on citrate indicator plates. *E. coli* cannot metabolize citrate because it lacks a transporter for citrate. The presence of the

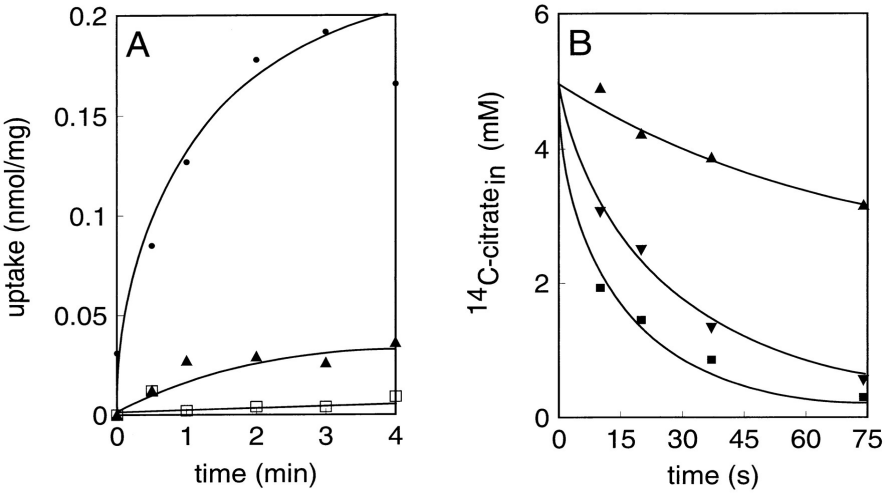

Figure 3. **Citrate transport catalyzed by CitP of *Lc. mesenteroides* expressed in *L. lactis*.** (A) Membrane vesicles isolated from *L. lactis* MG1363 expressing CitPlcm were fused to liposomes containing cytochrome *c* oxidase. Uptake of 4.5 μM [1,5-^{14}C]citrate was measured in the presence of no ionophores (triangle), valinomycin (circle) and nigericin (open square). The fused membranes were energized with the cytochrome *c*-TMPD-ascorbate electron donor system. (B) The same membrane vesicles were fused to liposomes and loaded with 5 mM [1,5-^{14}C]citrate, and subsequently diluted 100-fold into buffer containing no further additions (triangle), 5 mM citrate (filled square) or 5 mM D-lactate (wedge). In both experiments valinomycin and nigericin were present at concentrations of 1 μM and 0.5 μM, respectively. Uptake is expressed as nanomoles per milligram of membrane protein.

complete *citP* gene of *Lc. mesenteroides* on plasmid pNA1008 was demonstrated by a clear Cit$^+$ phenotype of the transformed *E. coli* cells on Christensen medium, which contained glucose as an additional energy and carbon source. Growth of the cells on minimal citrate medium (Simmons citrate agar) was poor.

A restriction map of pNA1008 was constructed (data not shown) and fragments were cloned into the phagemids pBluescript KS(+) and pBluescript KS(-). Single stranded DNA was isolated from both types of plasmids by infection with a helper phage, which allowed us to determine the nucleotide sequence of the inserts in both directions. Reconstruction of the sequence of the insert in pNA1008 revealed a 1,329-bp open reading frame. Two ATG initiation codons were preceded by a putative ribosomal binding site sequence GGAGA. No terminator of transcription could be detected behind the stop codon.

Sequence analysis. The *Lc. mesenteroides* 19D *citP* gene (*citPlcm*) is almost identical to the *citP* genes of *L. lactis* NCDO176 (*citPll*) and *Lc. lactis* NZ6070 (*citPlcl*). The *Lc. mesenteroides* gene is one and two codons longer than *L. lactis* and *Lc. lactis*, genes, respectively. In the translated amino acid sequence of the proteins the differences in length cluster in the region around position 260, and in the *Lc. mesenteroides* sequence one and two alanine residues are inserted compared to the other two sequences (Figure 2). Just preceding this position differences in the base sequences between the two *Leuconostoc* genes and the *Lactococcus* gene result in the mutations Asp256Lys and Ala257Ser. This region in the sequences is hydrophilic and in the structure predicted to be an interhelical loop; interhelical loops are often found to be quite variable in homologous membrane proteins (19, 40). Finally, conservative substitutions in the *Leuconostoc* and *Lactococcus* genes occur at positions 33 and 103, where Val is substituted for Ile and Met is substituted for Ile, respectively. The CitP proteins are homologous to the membrane potential generating malate transporter of *L. lactis* involved in malolactic fermentation and the Na^+-dependent citrate transporter CitS of *Klebsiella pneumoniae.* Together these transporters form the family of 2-hydroxy-carboxylate transporters (1).

Characterization of CitPlcm of *Lc. mesenteroides* expressed in *L. lactis*. Transport catalyzed by the cloned CitPlcm transporter expressed in *L. lactis* was characterized with

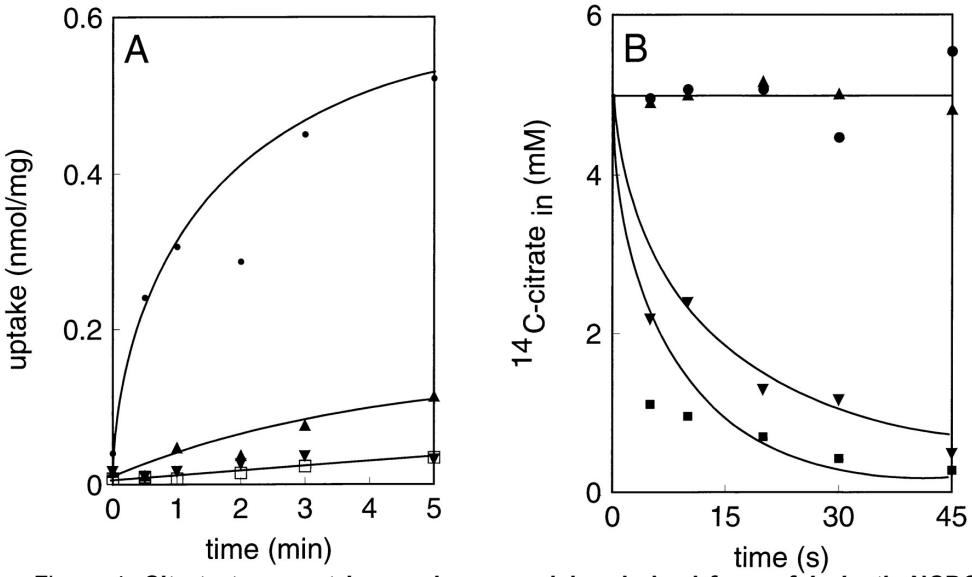

Figure 4. **Citrate transport in membrane vesicles derived from of *L. lactis* NCDO176.** The conditions were as described in the legend to Figure 3. (A) Uptake of 4.5 μM [1,5-^{14}C]citrate was measured in the presence of no ionophores (triangle), valinomycin (dot), and nigericin (open square) or in the absence of the cytochrome *c*-TMPD-ascorbate electron donor system (wedge). (B) Exchange: No further additions (triangle); 5 mM citrate (filled square); 5 mM D-lactate (wedge); 5 mM acetate (dot).

respect to energy coupling and heterologous exchange. The *citPlcm* gene of *Lc. mesenteroides* was cloned in expression vector pMB yielding pMBcitP in which expression is under control of the *citP* promotor of *L. lactis* (1, 22). The plasmid was transformed to *L. lactis* MG1363, a plasmid free strain that is not able to ferment citrate.

Cytoplasmic membranes with a right-side-out orientation were prepared from *L. lactis* harbouring plasmid pMBcitP, and the membranes were fused to proteoliposomes reconstituted with purified beef heart cytochrome *c* oxidase. This enzyme catalyzes electron transfer from reduced cytochrome *c* to molecular oxygen while it conserves the free energy of the reaction in the form of an electrochemical proton gradient across the membrane by pumping out protons. Generation of an electrochemical proton gradient or proton motive force across the membranes resulted in low citrate uptake; however, the level of citrate uptake was significantly higher than the level of uptake observed in the absence of the electron donor (Figure 3A). No uptake was observed with membranes of *L. lactis* MG1363 without plasmid pMBcitP. The proton motive force is composed of a pH gradient and the electrical membrane potential, which can be selectively dissipated by the ionophores nigericin (a K^+-H^+ antiporter) and valinomycin (a K^+ pore), respectively. Dissipation of the pH gradient completely prevented citrate uptake, while dissipation of the membrane potential resulted in a marked stimulation of citrate uptake. In conclusion, uptake of citrate in membrane vesicles is driven by the pH gradient and counteracted by the membrane potential.

Membrane vesicles prepared from *L. lactis* MG1363 expressing CitPlcm were fused to liposomes to improve the tightness of the membranes and loaded with 5 mM of ^{14}C-citrate. When the membranes were diluted into buffer without citrate at room temperature, a slow efflux of labeled citrate occurs in the first 75 s (Figure 3B). On the other hand, dilution into a buffer containing unlabeled citrate resulted in a rapid release of label from the membranes by homologous citrate-citrate exchange. Similarly, dilution into buffer containing an equivalent concentration of D-lactate demonstrated that the transporter catalyzes heterologous citrate-D-lactate exchange. The results show that the catalytic properties of the cloned CitPlcm of *Lc. mesenteroides* expressed in *L. lactis* are similar to the properties of the transporter in its native environment, the *Lc. mesenteroides* membrane (26).

Characterization of the citrate carrier CitPll of *L. lactis* NCDO176. CitPll of *L. lactis*

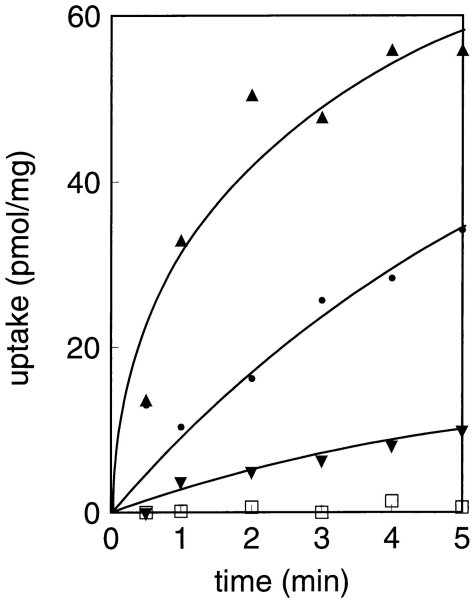

Figure 5. Functional expression of CitPlcm in *E. coli.* Cells of *E. coli* JM109(DE3) harbouring plasmid pSKcitP#3 (wedge, triangle, and dot) which encodes CitPlcm under control of the T7 polymerase, or control plasmid pBlueScript SK with no insert (open square) were grown in the presence of no IPTG (dot), 20 μM IPTG (triangle) and 50 μM IPTG (wedge). No IPTG was added to the control cells. The cells were resuspended to a OD_{660} of 5 and [1,5-^{14}C]citrate was added at a concentration of 4.5 μM. Uptake is expressed as picomoles per milligram of cell protein.

NCDO176 was characterized by using the same experimental approach as presented above for the transporter of *Lc. mesenteroides*. Thus, right-side-out membranes of *L. lactis* NCDO176 were fused to proteoliposomes containing cytochrome *c* oxidase and to liposomes and were assayed for proton motive force driven uptake and heterologous exchange, respectively. The results are presented in Figure 4 and are virtually the same as the results observed with CitPlcm of *Lc. mesenteroides*. The uptake activity was clearly stimulated by dissipation of the membrane potential and no uptake was observed when the pH gradient is dissipated (Figure 4A). The transporter catalyzed homologous exchange at a high rate while no efflux was observed and D-lactate was a substrate of the carrier in heterologous exchange (Figure 4B). Acetate is an end-product of citrate metabolism in lactic acid bacteria and has been implicated in the catalysis of CitPll of *L. lactis* (12). However, the data presented in Figure 4B clearly shows that acetate is not a substrate of the transporter. In conclusion, the citrate carriers of *L. lactis* and *Lc. mesenteroides* are mechanistically similar on the basis of these criteria.

Expression and characterization of CitPlcm in *E. coli*. Apparently, the data obtained with CitPll of *L. lactis* NCDO176 are at variance with the data in a previous report which described the characteristics of the cloned CitPll expressed in *E. coli.* An experiment similar to the the experiment shown in Figure 4A did not reveal stimulation of uptake when the membrane potential was dissipated by adding valinomycin (4). We repeated these experiments with *Lc. mesenteroides* CitPlcm to complete the series of experiments performed with the lactococcal and *Leuconostoc* CitPs.

From the initial stages of the cloning of the gene coding for CitPlcm it was clear that functional expression of the protein in *E. coli* was poor. In fact, the clone was detected by hybridization with *L. lactis citPll* DNA rather than by functional selection of plasmids containing the *Lc. mesenteroides* DNA fragments on citrate indicator plates. We constructed a series of plasmids containing the *citPlcm* gene under control of different promoters and transformed them into different strains of *E. coli* that were grown in minimal and rich media with and without induction, but the functional expression of CitPlcm was invariably low. Attempts to increase the expression of CitP in the membrane resulted in inhibition of growth of the cells, a phenomenon often observed when a membrane protein is overexpressed. In the experiment shown in Figure 5 the *citP* gene is cloned behind the T7 promoter and transformed to *E. coli* JM109(DE3) that contains a chromosomal copy of the T7 polymerase under control of an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter (36). In the absence of inducer, a small amount of the polymerase was synthesized due to leakage of the promoter, and a low but significant uptake of citrate into the cells was observed. Addition of increasing

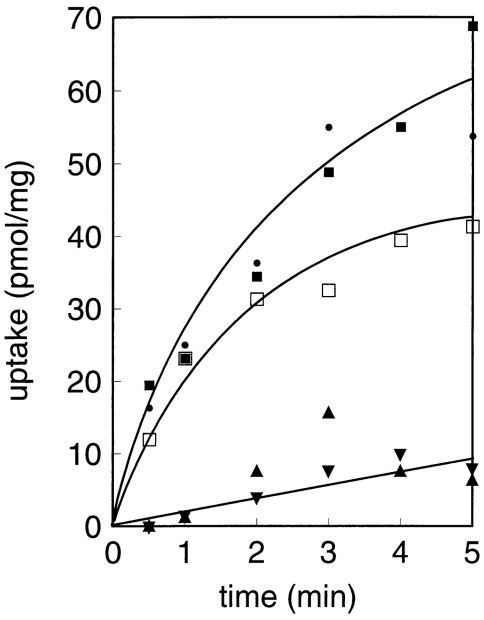

Figure 6. Energy coupling to citrate transport catalyzed by CitPlcm of *Lc. mesenteroides* expressed in *E. coli*. Right-side-out membrane vesicles were prepared from *E. coli* JM101 cells harbouring plasmid pSKcitP#56 which encodes CitPlcm under control of the Lac promoter. The membranes were resuspended at a concentration of 0.8 mg of protein per mL and were not energized (triangle) or energized (dot, wedge, filled square, and open square) by the addition of 10 mM potassium ascorbate and 0.2 mM PMS. Uptake of [1,5-^{14}C]citrate at a concentration of 4.5μM was measured with no further additions (dot) and in the presence of 0.5 μM nigericin (wedge), 0.125 μM valinomycin (filled square) and 1 μM valinomycin (open square).Uptake is expressed as picomoles per milligram of membrane protein.

concentrations of the inducer IPTG results, at first, in an increase of the expression of CitP, but unfortunately, at higher IPTG concentrations the uptake decreased dramatically. The decreased uptake activity was parallelled by a decreased growth rate of the cells, showing the toxicity of the CitP protein in *E. coli*.

The low uptake activity observed in whole cells might be a consequence of the mechanism by which CitPlcm catalyzes citrate transport. Resting cells of *E. coli* maintain a high membrane potential that is expected to counteract transport. Valinomycin cannot be used to selectively dissipate the membrane potential since added ionophore is scavenged by the outer membrane. Therefore, cytoplasmic membrane vesicles were prepared from the cells expressing CitPlcm which allow manipulation of the composition of the proton motive force. Nevertheless, Figure 6 shows that the uptake activity was at least two orders of magnitude lower than the uptake activity observed with vesicles prepared from cells expressing, for instance, the Na^+-dependent citrate transporter of *Klebsiella pneumoniae*, a transporter that is homologous to the CitPs of lactic acid bacteria (39). Surprisingly, but consistent with the results obtained previously with the *L. lactis* CitPll expressed in *E. coli*, no stimulation of uptake was observed when the membrane potential was selectively dissipated with valinomycin. At higher concentrations, valinomycin was slightly inhibitory. Dissipation of the pH gradient completely prevented any uptake of citrate above background levels. Together, the results of these experiments suggest that citrate uptake by CitPlcm expressed in *E. coli* is catalyzed by an electroneutral proton symport mechanism.

DISCUSSION

In lactic acid bacteria the ability to ferment citrate is associated with endogenous plasmids that contain the gene that codes for the system responsible for uptake of citrate into the cells. Genes coding for CitPs have been described for strains of *L. lactis* and *Lc. lactis* (4, 41). The cloned lactococcal CitP is coded on a 7.9-kb plasmid which appears to be present in all citrate fermenting *Lactococcus* strains analyzed so far (15). In *Leuconostoc* strains the plasmids that are indispensable for citrate metabolism are larger and more variable in size for the different strains (41). Consistent with this observation, in this study we found that a *citP* gene is present on a 22.4-kb endogenous plasmid of *Lc. mesenteroides* 19D. A strong signal was obtained when plasmid DNA isolated from *Lc. mesenteroides* was hybridized with a probe derived from the *L. lactis citP* gene, and subsequent cloning and sequencing revealed an open reading frame whose sequence was almost identical to the other two known *citP* nucleotide sequences. Clearly, although the transporters are almost identical, the genetic context of the *citP* genes is

different in *Lactococcus* and *Leuconostoc* strains, and it has been shown that mechanisms that control the expression of the genes are different (22, 24, 28).

Citrate metabolism in *Lc. mesenteroides* results in the generation of a proton motive force by a secondary mechanism in which the CitP is responsible for the generation of the membrane potential (26, 27). Evidence that the *citP* gene of *Lc. mesenteroides* described in this report codes for the membrane potential generating transporter comes from (i) loss of the Cit^+ phenotype correlates with the loss of the 22-kb plasmid; (ii) loss of the 22-kb plasmid correlates with the loss of citrate uptake in membrane vesicles (26); and (iii) the characteristics of citrate transport in membrane vesicles derived from *L. lactis* MG1363 expressing *citPlcm* are similarto the characteristics observed with membrane vesicles derived from *Lc. mesenteroides* (Figure 3).

The physiological mode of transport of CitP of *Lc. mesenteroides* is precursor-product exchange. Divalent citrate is exchanged for monovalent lactate which is the product of citrate metabolism during glucose-citrate cometabolism (27). In membrane vesicles in which the citrate metabolic enzymes are absent and no lactate is formed, CitP functions as a proton/symporter that translocates divalent citrate with a single proton (26). Both modes of transport are counteracted by the membrane potential and together they are diagnostic for secondary transporters that function in a secondary proton motive force generating pathway. The unidirectional symport and the exchange reaction show that the transporters function as membrane potential generator and precursor-product exchanger, respectively. When these criteria were used, the *citP* gene product described in this paper could be identified as the CitP that operative in citrate metabolism in *Lc. mesenteroides* (Figure 3). Moreover, by using the same criteria, it was shown that the transporter of *L. lactis*, CitPll, catalyzes citrate transport via the same mechanism (Figure 4). The results show that neither the few differences in the amino acid sequences of CitPlcm and CitPll nor the differences in cytoplasmic membrane composition of *Lc. mesenteroides* and *L. lactis* affect the mechanism of the two transporters.

The results in previous experiments performed with the cloned lactococcal CitPll expressed in *E. coli* suggested that there is a proton motive force driven transport mechanism, which was considered evidence that energy dependent citrate uptake by CitPll occurs in *L. lactis* (4). The initial rate of citrate uptake in membrane vesicles derived from *E. coli* expressing CitPll was reduced rather than stimulated when the membrane potential was dissipated, indicating that the membrane potential does not counteract transport. In this study we confirm these observations with the cloned CitPlcm of *Lc. mesenteroides*. Under the conditions of the experiments in a buffer at pH 6, the ascorbate-PMS electron donor system has been shown to generate a significant membrane potential in *E. coli* membrane vesicles (37). Nevertheless, dissipation of the membrane potential did not result in stimulation of the uptake of citrate (Figure 6). Apparently, the results with the cloned CitPll and CitPlcl expressed in *E. coli* are at variance with the results obtained with the same transporters in their native environment (Figure 3 and 4). A similar observation has been made with the glutamate transporter GltT of the thermophilic bacterium *B. stearothermophilus.* Studies with membrane vesicles derived from *B. stearothermophilus* showed that GltT translocates glutamate in symport with two cations, a proton and a Na^+ ion. Surprisingly, expression of GltT in *E. coli* resulted in the complete loss of the Na^+-dependency (8, 38). These observations were explained by suggesting that the lipid environment plays a role in the cation specificity of the transporter.

In a secondary metabolic energy generating pathway, the two components of the proton motive force are generated in separate steps; the membrane potential is generated in the transport step (electrogenic citrate-lactate exchange) and the pH gradient is a consequence of the consumption of a cytoplasmic proton in the decarboxylation step (oxaloacetate decarboxylation). Nevertheless, it can be demonstrated that the two events are coupled indirectly (20). Thus, membrane potential generation by citrate uptake via CitPll of *L. lactis* necessarily results in generation of a pH gradient across the cytoplasmic membrane when a proton is consumed in the oxaloacetate decarboxylation step. Therefore, the identical

mechanisms of the CitPs of *L. lactis* and *Lc. mesenteroides* demonstrated in this study strongly suggest that citrate metabolism *L. lactis* is a secondary metabolic energy generating process.

REFERENCES

1. **Bandell, M., V. Ansanay, N. Rachidi, S. Dequin, and J. S. Lolkema.** 1997. Membrane potential generating malate (MleP) and citrate (CitP) transporters of lactic acid bacteria are homologous proteins. Substrate specificity of the 2-hydroxy-carboxylate transporter family. J. Biol. Chem. **272:**18140-18146.
2. **Christensen, W. B.** 1949. Hydrogen sulfide production and citrate utilization in the differentiation of enteric pathogens and coliform bacteria. Res Bull, Weld Country Health. Department, Greeley, Colorado, **1:**3-16.
3. **Cogan, T. M.** 1987. Co-metabolism of citrate and glucose by *Leuconostoc* ssp.: effects on growth, substrates and products. J. Appl. Bacteriol. **63:**551-558.
4. **David, S., M. E. Van Der Rest, A. J. M. Driessen, G. Simons, and W.M. De Vos.** 1990. Nucleotide sequence and expression in *Escherichia coli* of the *Lactococcus lactis* citrate permease gene. J. Bacteriol. **172:**5789-5794.
5. **De Man, J. C., M. Rogosa, and M. E. Sharpe.** 1960. A medium for the cultivation of *Lactobacilli*. J. Appl. Bacteriol. **23:**130-135.
6. **Driessen, A. J. M., W. de Vrij, and W. N. Konings.** 1985. Incorporation of beef heart cytochrome *c* oxidase as a proton motive force generating mechanism in bacterial membrane vesicles. Proc. Natl. Acad. Sci. USA **82:**7555-7559.
7. **Driessen, A. J. M., L. Brundage, J. P. Hendrick, E. Schiebel, and W. Wickner.** 1991. Preprotein translocase of Escherichia coli: solubilization, purification and reconstitution of the integral membrane subunits SecY/E. Meth. Cell. Biol. **34**:147-165.
8. **Gaillard, I., D-J. Slotboom, J. Knol, J. S. Lolkema, and W.N. Konings.** 1995. Purification and reconstitution of the glutamate carrier GltT of the thermophilic bacterium *Bacillus stearothermophilus*. Biochemistry **35:**6150-6156.
9. **Gasson, M.** 1983. Plasmids complements of *Streptococcus lactis* NCDO712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1-9
10. **Holo, H. and I. F. Ness.** 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis,* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55:**3119-3123.
11. **Hugenholtz, J.** 1993. Citrate metabolism in lactic acid bacteria. FEMS Microbiol. Rev. **12:**165-178.
12. **Hugenholtz, J., L. Perdon, and T. Abee.** 1993. Growth and energy generation by *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* during citrate metabolism. Appl. Environ. Microbiol. **59:**4216-4222.
13. **Kaback, H.R.** 1971. Bacterial membranes. Meth. Enzymol. **22**:99-120.
14. **Kempler, G. M. and L. L. McKay.** 1980. Improved medium for detection of *Streptococcus lactis* ssp *diacetylactis*. Appl. Environ. Microbiol. **39:**926-927.
15. **Kempler, G.M., and L.L. McKay.** 1981. Biochemistry and genetics of citrate utilization in *Streptococcus lactis* ssp *diacetylactis*. J. Dairy Sci. **64:**1527-1531.
16. **Konings, W. N., J. S. Lolkema, and B. Poolman.** 1995. The generation of metabolic energy by solute transport. Arch. Microbiol. **164:**235-242.
17. **Lamoureux, M., H. Prevost, J. F. Cavin, and C. Diviès.** 1993. Recognition of *Leuconostoc oenos* strains by the use of DNA restriction profiles. Appl. Microbiol. Biotechnol. **39:**547-552.
18. **Lin J., P. Schmitt, and C. Divies.** 1991. Characterization of a citrate-negative mutant of *Leuconostoc mesenteroides* subsp. *mesenteroides*: metabolic and plasmidic properties. Appl. Microbiol. Biotech. **34:**628-631.
19. **Lolkema, J. S., G. Speelmans, and W. N. Konings.** 1994. Na^+-coupled versus H^+-coupled energy transduction in bacteria. Biochim. Biophys. Acta **1187**:211-215.
20. **Lolkema, J. S., B. Poolman, and W. N. Konings.** 1995. Role of scalar protons in metabolic energy generation in Lactic Acid Bacteria. J. Bioenerg. Biomembr. **27:**467-473.
21. **Lolkema, J. S., B. Poolman, and W. N. Konings.** 1996. Secondary transporters and metabolic energy generation in bacteria, p. 229-260. *In* W. N. Konings, H. R. Kaback and J. S. Lolkema (Eds.), Handbook of biological physics. Elsevier Science, Amsterdam.
22. **López de Felipe, F., C. Magni, D. de Mendoza, and P. López.** 1995. Citrate utilization gene cluster of the *Lactococcus lactis* biovar *diacetylactis:* organization and regulation of expression. Mol. Gen. Genet. **246:**590-599.
23. **Lowry, O. H., N. J. Rosebrough, A. J. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193:**265-275.
24. **Magni, C., F. López de Felipe, F. Sesma, P. López, and D. de Mendoza.** 1994. Citrate transport in *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis.* Expression of the citrate permease P. FEMS Microbiol. Lett. **118:**75-82.
25. **Magni, C., P. López, and D. de Mendoza.** 1996. The properties of citrate transport catalyzed by CitP of *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis*. FEMS Microbiol. Lett. **142:**265-269.

26. **Marty-Teysset, C., J. S. Lolkema, P. Schmitt, C. Divies, and W. N. Konings.** 1995. Membrane potential generating transport of citrate and malate catalyzed by CitP of *Leuconostoc mesenteroides.* J. Biol. Chem. **270:**25370-25376.
27. **Marty-Teysset, C., C. Posthuma, J. S. Lolkema, P. Schmitt, C. Divies, and W. N. Konings,** 1996. Proton motive force generation by citrolactic fermentation in *Leuconostoc mesenteroides*. J. Bacteriol. **178:**2178-2185.
28. **Marty-Teysset, C., J. S. Lolkema, P. Schmitt, C. Divies, and W. N. Konings, W. N.** 1996. The citrate metabolic pathway in *Leuconostoc mesenteroides.* Expression, amino acid synthesis and α-ketocarboxylate transport. J. Bacteriol. **178:**6209-6215.
29. **Mayer, D. L., M. J. Hope, and P. R. Cullis. 1986.** Vesicles of variable sizes produced by a rapid extrusion procedure. Biochim. Biophys. Acta **858:**161-168.
30. **O'Sullivan, D.J. and T. R. Klaenhammer.** 1993. Rapid mini-preparation of high--quality DNA from *Lactococcus* and *Lactococcus spp*. Appl. Environ. Microbiol. **59:**2730-2733.
31. **Otto, R., R. G. Lageveen, H. Veldkamp, and W. N. Konings.** 1982. Lactate efflux-induced electrical potential in membrane vesicles of *Streptococcus cremoris*. J. Bacteriol. **149**:733-738.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning : a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.
33. **Simmons, J. S.** 1926. A culture medium for differentiating organisms of typhoidcodon aerogenes and for isolating of certain fungi. J. Infect. Dis. **39:**209-214.
34. **Southern, E.M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503-517.
35. **Starrenburg, M. and J. Hugenholtz.** 1991. Citrate fermentation by *Lactococcus* and *Leuconostoc* spp. Appl. Environ. Microbiol. **57:**3535-3540.
36. **Studier, F.W. and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113-130.
37. **Ten Brink, B. and W. N. Konings.** 1980. Generation of an electrochemical proton gradient by lactate efflux in *Escherichia coli* membrane vesicles. Eur. J. Biochem. **111**:59-66.
38. **Tolner, B., T. Ubbink-Kok, B. Poolman, and W. N. Konings.** 1995. Cation-selectivity of the L-Glutamate transporters of *Escherichia coli, Bacillus stearothermophilus* and *Bacillus caldotenax.* Mol. Microbiol. **18**:123-133.
39. **Van der Rest, M.E., R. M. Siewe, T. Abee, E. Schartz, and D. Oesterhelt.** 1992. Nucleotide sequence and functional properties of a sodium-dependent citrate transport system from *Klebsiella pneumoniae.* J. Biol. Chem. **267**:8971-8976.
40. **Van Geest, M. and J. S. Lolkema.** 1996. Membrane topology of the Na^+-dependent citrate transporter of *Klebsiella pneumoniae*. Evidence for a new structural class of secondary transporters. J. Biol. Chem. **271:**25582-25589.
41. **Vaughan, E. E., S. David, A. Harrington, C. Daly, G. F. Fitzgerald, and W. M. De Vos.** 1996. Characterization of plasmid-encoded citrate permease (citP) genes from *Leuconostoc* species reveals high sequence homology conservation with the *Lactococcus lactis* citP gene. Appl. Environ. Microbiol. **61:**3172-3176.

Chapter 3

Membrane potential generating malate (MleP) and citrate (CitP) transporters of lactic acid bacteria are homologous proteins.

SUBSTRATE SPECIFICITY OF THE 2-HYDROXYCARBOXYLATE TRANSPORTER FAMILY

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SUMMARY

Membrane potential generation via malate-lactate exchange catalyzed by the malate carrier (MleP) of *Lactococcus lactis*, together with the generation of a pH gradient via decarboxylation of malate to lactate in the cytoplasm, is a typical example of a secondary proton motive force-generating system. The *mleP* gene was cloned, sequenced, and expressed in a malolactic fermentation-deficient *L. lactis* strain. Functional analysis revealed the same properties as observed in membrane vesicles of a malolactic fermentation-positive strain. MleP belongs to a family of secondary transporters in which the citrate carriers from *Leuconostoc mesenteroides* (CitP) and *Klebsiella pneumoniae* (CitS) are found also. CitP, but not CitS, is also involved in membrane potential generation via electrogenic citrate-lactate exchange. MleP, CitP and CitS were analyzed for their substrate specificity. The 2-hydroxycarboxylate motif HO-CR_2-COO^-, common to the physiological substrates, was found to be essential for transport although some 2-oxocarboxylates could be transported to a lesser extent. Clear differences in substrate specificity among the transporters were observed because of different tolerances toward the R substituents at the C2 atom. Both MleP and CitP transport a broad range of 2-hydroxycarboxylates with R substituents ranging in size from two hydrogen atoms (glycolate) to acetyl and methyl groups (citramalate) for MleP and two acetyl groups (citrate) for CitP. CitS was much less tolerant and transported only citrate and at a low rate citramalate. The substrate specificities are discussed in the context of the physiological function of the transporters.

INTRODUCTION

The electrochemical gradient of protons across the cytoplasmic membrane is a major store of free energy in the bacterial cell. Usually, the proton motive force (PMF) is generated by translocation of protons against the gradient across the cell membrane which results in the two components of the PMF, a membrane potential and a pH gradient. Proton pumping is catalyzed by primary transport systems at the expense of some source of chemical energy or light. In certain anaerobes a different mechanism of PMF generation has evolved which involves the action of secondary transporters rather than primary ion pumps and, therefore, is termed secondary PMF generation (for reviews see Refs. 1 and 2). An example of such a system is the malolactic fermentation pathway found in several lactic acid bacteria (3-5). In *Lactococcus lactis* uptake of divalent malate is coupled to the exit of its decarboxylation product, monovalent lactate (precursor-product exchange) which leads to the formation of a membrane

potential of physiological polarity. Furthermore, the intracellular decarboxylation of malate catalyzed by malolactic enzyme consumes a cytosolic proton which results in a pH gradient over the cell membrane. The combined activities of electrogenic exchange and proton-consuming decarboxylation result in a proton motive force that is sufficiently high to drive ATP synthesis via the F_0F_1 ATPase (3). Similar pathways have been described for a number of other substrates (6-8). Recently, a more complex system termed citrolactic fermentation, was found in *Leuconostoc mesenteroides,* another lactic acid bacterium. Similar to the malolactic fermentation pathway a secondary transporter catalyzes the uptake of divalent citrate in exchange for monovalent lactate, but the pathway in the cytosol converting citrate into lactate requires three different enzymes and is coupled to glucose metabolism (9, 10).

The membrane potential-generating secondary transporters involved in malolactic fermentation and citrolactic fermentation, MleP and CitP, respectively, differ from "usual" secondary transporters in two aspects, (i) they translocate net negative charge across the membrane and (ii) they catalyze efficient heterologous exchange of two structurally related substrates (the precursor and the product). Functionally, MleP and CitP are quite similar because lactate is a substrate of both, and moreover, it was shown that malate is a substrate of CitP as well (9). The structural gene coding for CitP was cloned from different organisms (11, 12) and shown to be homologous to the Na^+-dependent citrate carrier CitS of *Klebsiella pneumoniae* (13). CitS is a "usual" secondary transporter driven by the PMF and sodium ion motive force (14-16). Recently, CitS was shown to represent a new structural class of secondary transporters with a nine helix bundle motif (17).

Here, we report the cloning and sequencing of the *mleP* gene coding for the malate transporter of *L. lactis* which is involved in malolactic fermentation. In line with the functional similarities of MleP and CitP the gene was found to be homologous to the *citP* and *citS* genes. The three proteins are part of a family of secondary transporters in which both metabolic energy-dissipating (CitS) and -generating (CitP and MleP) members are found. Essential for MleP and CitP is the ability to transport two differently charged, but structurally related molecules, which suggests a wide substrate specificity. It is shown that a broad range of 2-hydroxycarboxylates can be transported by members of the family. In line with its physiological function, the substrate specificity of CitS is much more restricted than observed for the two precursor-product exchangers.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. *L. lactis* strains IL1403 and IL1441 are wild type malate-fermentating strains; strains MG1363 and LL108 do not ferment malate. Strain IL1441 is a streptomycin resistant derivative of IL1403 (18). Strain LL108, kindly provided by K. Leenhouts, is a chloramphenicol-resistant derivative of MG1363 with multiple copies of the *repA* gene inserted in its chromosome which results in an increase in plasmid copy number (19). *L. lactis* NCDO176 is a wild type citrate fermenting strain obtained from the Dutch Institute of Dairy Research (Ede, The Netherlands). The *L. lactis* strains were grown in closed serum bottles without shaking in M17 broth (Difco) supplemented with 0.5 % (w/v) glucose and at 30 °C. Concentrations of 5 μg/mL erythromycin and 5 μg/mL chloramphenicol were used when indicated. *Leuconostoc mesenteroides* ssp. *mesenteroides* 19D was grown at 30 °C in MRS medium without acetate and Tween and with 0.5 % ammonium citrate (20). *Escherichia coli* strains DH5α, BL21(DE3) and MC1061 were grown aerobically at 37 °C in Luria Broth supplemented with 100 μg/mL carbenicillin or 10 μg/mL chloramphenicol when indicated.

Recombinant DNA techniques. Standard DNA manipulation techniques were carried out essentially as described by Sambrook *et al*. (21). *L. lactis* genomic DNA was isolated as described by Simon *et al.* (22). *L. lactis* and *Lc. mesenteroides* plasmid DNA was isolated as described by Leenhouts et al. (23). *L. lactis* was transformed by electroporation as described by Holo and Nes (24). For sequencing, plasmids were transformed to and propagated in *E. coli* DH5α. Nucleotide sequences were determined on a Vistra 725 or Applied Biosystems 373A automated sequencer.

Cloning and sequencing of the mleP gene. Plasmid p153A has been described before (25) and was shown to contain *mleS* encoding malolactic enzyme and the 5' part of a second open reading frame ORF2, presumable *mleP* encoding the malate transporter. A 3-kb fragment obtained by *Bam*HI digestion of chromosomal DNA isolated from *L. lactis* IL1441 overlapped with the insert in p153A. The fragment was cloned into the unique *Bam*HI

restriction site of plasmid pUC18 (26) yielding p191A. Unidirectional sets of nested deletions of p191A were constructed using exonuclease III (Pharmacia). Truncated fragments were sequenced using vector-specific primers after which internal primers were designed to sequence the remaining regions of each strand. The insert contained the first 1,068 bp of ORF2. To clone the missing 3' end of ORF2, an *Eco*RI/*Bam*HI fragment of p191A containing the final 900 base pairs of the ORF2 fragment was used to probe a *Hind*III digest of chromosomal DNA. The sequence of a 2.5-kb fragment that hybridized with the probe was determined using inversed polymerase chain reaction (PCR). Genomic DNA of *L. lactis* IL1441 digested with *Hind*III was ligated with T4 DNA ligase under conditions that favored the formation of monomeric circles. The circular fragments were used as a template for a PCR using oligonucleotides based on the sequence of the *Eco*RI/*Bam*HI fragment of p191A. The primer sequences were 5'-TGGCAGGTATTGGTCTTG-3' and 5'-CCATACCACCTGACATCAT-3'. A 2-kb fragment presenting a head to tail structure was amplified and cloned into pGEM-T (Invitrogen) to give vector pPNJ. The missing 0.4-kb fragment of ORF2 on pPNJ was sequenced on both strands. ORF2 was amplified from genomic DNA using a forward primer based on the sequence of p153A (5'-ATCTCGAGATGAAAAAACTTAAAGAA-3') and a reverse primer based on the sequence of pPNJ (5'-GATAGGCGATATAGTCCC-3') and ligated into pGEM-T, yielding pPME. The sequence of ORF2 reconstructed from all the fragments was confirmed by sequencing one strand of the pPME insert.

Construction of expression vectors. An expression vector was constructed containing a promoter region that is located in front of the *citP* gene on the 7.9-kb plasmid of *L. lactis* NCDO176 (27). The 1,572-base pair region was amplified from the plasmid by PCR. The forward primer, 5'-GCTTAGAATTCCTTGCTATCAGTATGTC-3' introduced an *Eco*RI restriction site in the fragment. The reverse primer 5'-GGTGGATCCA**CAT**GTTTCTATCTCCATTTATATC-3' overlapped with the *citP* start codon and introduced a unique *Afl*III site (ACATGT) around the start codon and a *Bam*HI site at the end. Bases introducing mutations are underlined and the start codon is indicated in bold. The PCR product was digested with *Eco*RI and *Bam*HI and ligated into plasmid pGK13 (28) digested with the same enzymes. The resulting plasmid pMB contains the *L. lactis citP* promoter region followed by a ribosomal binding site and a *Afl*III restriction site around the start codon. Downstream of the *Afl*III restriction site a number of unique restriction sites are present for cloning purposes.

The gene coding for the malate transporter *mleP* was amplified by PCR from chromosomal DNA isolated from *L. lactis* IL1403. The forward primer 5'-TCTTAATCC**ATG**GGAAAAAAACTTAAAG AAACGAAAATATCGG-3' generated a *Nco*I site around the start codon while the reverse primer 5'-TGCTCTAGA**TTA**CGCGTATACAAAGAATCGGATAAGAATTCCACC-3' generated a *Xba*I site downstream of the stop codon. Start and stop codons are indicated in bold. Similarly, the gene coding for the citrate transporter *citP* was amplified from an endogenous plasmid preparation of *Lc. mesenteroides* while creating unique *Nco*I and *Xba*I restriction sites around the start codon and downstream of the stop codon, respectively. The forward primer was 5'-GATAGAACC**ATG**GTGAATCACCCGCATTC-3' and the reverse primer 5'-CTTTAAATATCTAGA**TTA**CTTCATG-3'. The amplified fragments were digested with *Nco*I and *Xba*I and ligated into the *Afl*III and *Nhe*I sites of pMB which have compatible overhangs. The resulting vectors pMB*mleP* and pMB*citP* code for *mleP* and *citP* under control of the *citP* promoter. In case of MleP, the cloning procedure resulted in a Gly insertion after position 1, and the C-terminal Tyr residue was replaced by Val-Tyr-Ala. In CitP Met2 was replaced by Val. The sequence of the inserts was confirmed by automated sequencing.

Preparation of membrane vesicles and hybrid membranes. Cells of *L. lactis* MG1363 or LL108 expressing either MleP or CitP were harvested at the end of the exponential growth phase at an OD_{660} of 0.8, washed with 50 mM potassium phosphate pH 7.0, resuspended in the same buffer at an OD_{660} of 500 and, subsequently, rapidly frozen in liquid nitrogen until use. Right-side-out membrane vesicles were prepared by the osmotic shock lysis procedure essentially as described by Otto *et al*. (29). The membranes were fused to liposomes or to proteoliposomes containing beef heart cytochrome *c* oxidase (COVs), essentially as described by Driessen *et al*. (30). Liposomes consisted of a mixture of the purified *E. coli* lipids and egg yolk phosphatidylcholine at a ratio 3:1. *E. coli* lipids were purified by successive washing of an *E. coli* extract (Avanti Polar Lipids) with acetone and diethyl ether after which the concentration was determined as described by Driessen *et al*.(31). Cytochrome *c* oxidase isolated from beef heart mitochondria was reconstituted into liposomes by detergent dialysis. Liposomes or COVs were fused with the membrane vesicles of *L. lactis* at a ratio of 10 mg of lipid and 1 mg of protein by a single freeze/thaw step (30). The buffer contained 50 mM potassium phosphate, pH 6. In case of fusion with liposomes 5 mM L-malate or citrate were included in the buffer in order to load the vesicles. The resulting hybrid membranes were made unilamellar by subsequent extrusion through 400- and 200-nm pore size polycarbonate filters (32). Hybrid membranes were concentrated by ultra centrifugation at 250,000 x g for 20 min at 10 °C. Right-side-out membrane vesicles of *E. coli* strain BL21(DE3) expressing CitS from pSKΔL*citS* (17) were prepared by the osmotic lysis procedure essentially as described by Kaback (33) with the following modification. Spheroplasts were lysed in 50 mM potassium phosphate pH 7 containing 5 mM potassium citrate to load the vesicles with citrate. All subsequent steps were done in the presence of 5 mM potassium citrate. Before use the membrane vesicles were washed once in 15 volumes of potassium phosphate pH 6 containing 5 mM citrate and appropriate concentrations NaCl and KCl and concentrated by centrifugation for 20 minutes in a Eppendorf table top centrifuge operated at full speed. Protein concentrations were determined as described by Lowry *et al*. (34).

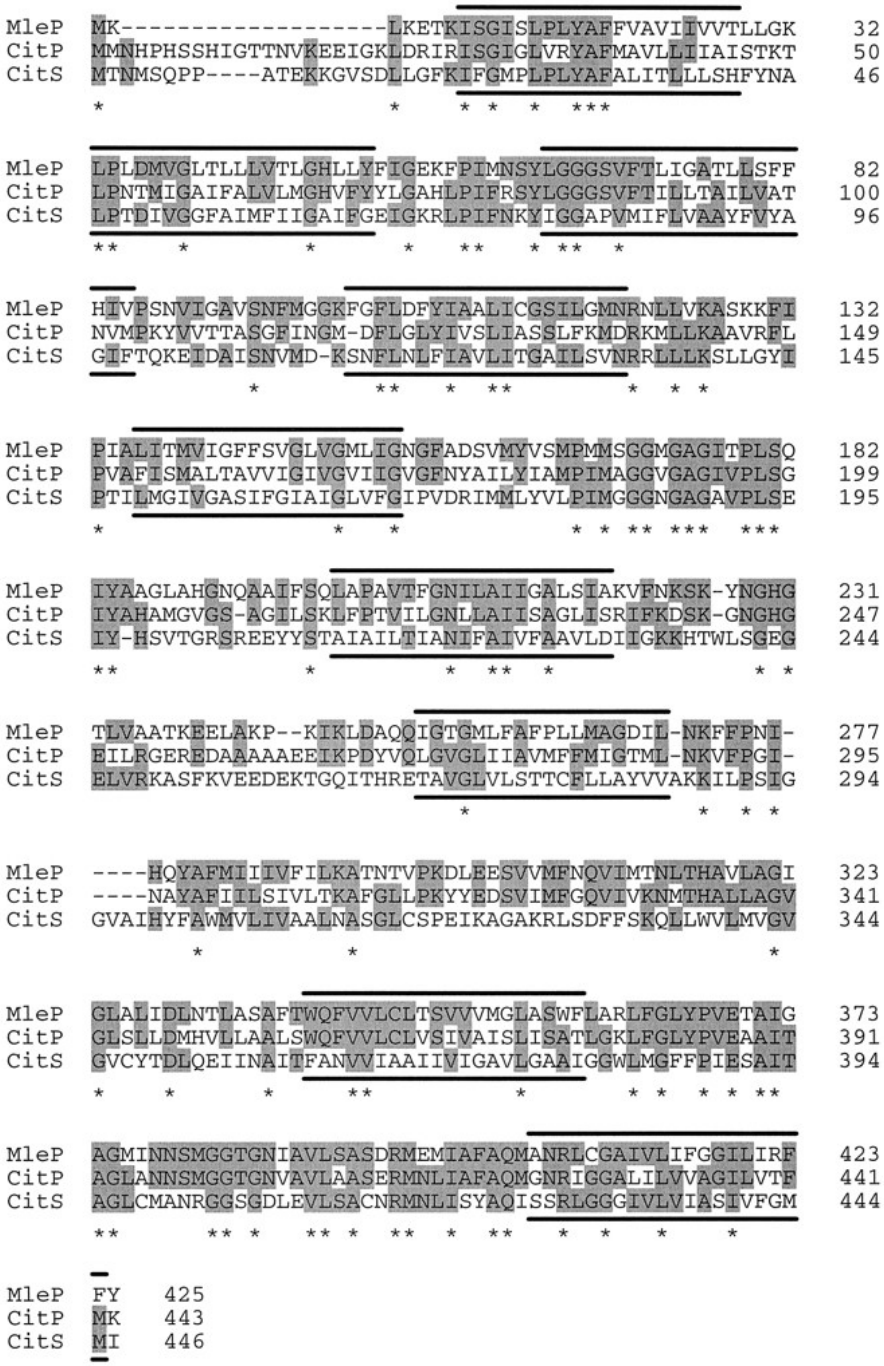

Figure 1. **Alignment of the primary sequences of MleP, CitP and CitS.** Identical residues are printed in gray boxes. Residues conserved in all three transporters are indicated by an asterix. Putative transmembrane segments (17) are indicated by bars.

Transport Assays *(i) PMF driven uptake in hybrid membranes.* The experiments were performed in 50 mM potassium phosphate pH 6.0 under a flow of water saturated air and continuous stirring at 30 EC. Membrane vesicles fused with COVs were incubated for 1 min in the presence of 200 µM N,N.N=,N=-tetramethyl-p-phenylenediamine (TMPD), 20 µM cytochrome *c* (horse heart, Sigma) and 10 mM potassium ascorbate. The assay volume was 100 µL and the membrane protein concentration 0.6-0.8 mg/mL. Valinomycin and nigericin were used at concentrations of 1 µM and 0.5 µM, respectively. L[1,4(2,3)^{14}C]malate or [1,5-^{14}C]citrate was added at concentrations of 13.1 and 4.4 µM, respectively. Uptake was stopped at different time intervals by adding 2 mL ice-cold 0.1 M LiCl to a sample and rapid filtration over 0.45 µm pore size cellulose nitrate filters (Schleicher & Schuell). Filters were rinsed once with 2 mL of ice-cold 0.1 M LiCl and transferred to scintillation vials, and the internallized radioactivity was determined.

(ii) Exchange in Membrane Vesicles. Membrane vesicles of *L. lactis* LL108 fused to liposomes and *E. coli* right-side-out membrane vesicles preloaded with 5 mM L-malate or citrate were concentrated by centrifugation. Strain LL108 was used for this assay because of the higher expression of MleP and CitP in this strain. Concentrated hybrid membranes were incubated in 50 mM potassium phosphate pH 6 with 50 µM nigericin with 72.5 µM [1,5-^{14}C]citrate or 186.7 µM L[1,4(2,3)^{14}C]malate for 30 min at room temperature. When indicated, 100

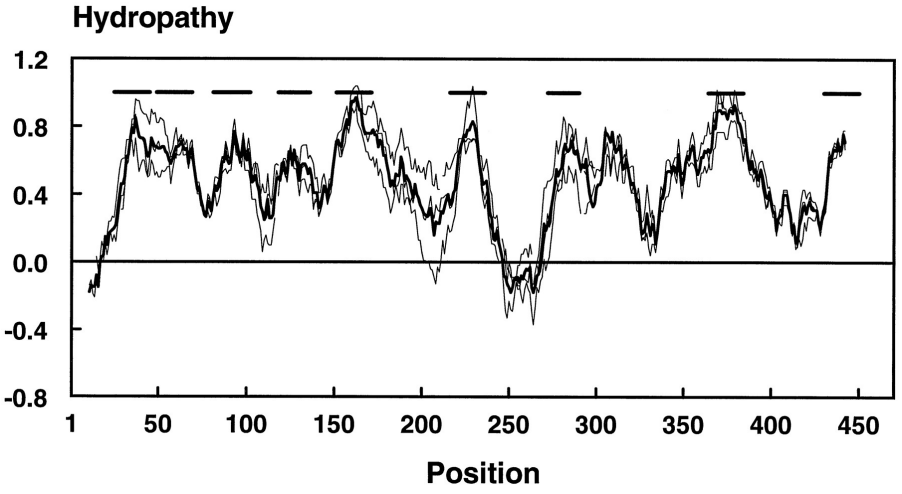

Figure 2. **Hydropathy profile of MleP, CitP and CitS.** The individual profiles (thin) and average profile (bold) were calculated with a window of 21 residues and the normalized hydrophobicity scale of Kyte (42). Putative transmembrane segments are indicated by bars (17).

μM valinomycin or 100 mM KSCN were present in the assay mixture. Similarly, the concentrated *E. coli* membranes were incubated in 50 mM potassium phosphate pH 6 in the presence of 100 μM valinomycin and 50 μM nigericin with 217.5 μM [1,5-^{14}C]citrate and the appropriate concentrations of NaCl and KCl for 2 h. Aliquots of 2 μL were diluted 100-fold into buffer of 20 C containing various substrates at a concentration of 5 mM. The buffer contained 100 mM KCl in the case the membranes where preloaded with KSCN. Final membrane protein concentrations in the assays were 0.1-0.15 mg/mL and 0.23 mg/mL for *L. lactis* hybrid membranes and *E. coli* membranes, respectively. Samples were stopped and processed as described above. The data was fitted to an exponential decay. Within one set of experiments, the zero time point was determined from the curve representing efflux which is slow enough to allow a linear back extrapolation. The infinite time point was estimated from curves representing rapid exchange, usually homologous exchange.

Chemicals. [1,5-^{14}C]citrate (115mCi/mmol) and L[1,4(2,3)^{14}C]malate (51 mCi/mmol) were obtained from Amersham International (Buckinghamshire, UK). Oligonucleotides were obtained from Eurosequence (Groningen, The Netherlands) and from Eurogentec (Seraing, Belgium). All other compounds were obtained from commercial sources.

RESULTS

Cloning and sequencing of mleP. In a previous paper the cloning and sequencing was reported for the gene *mleS* encoding malolactic enzyme, the decarboxylase in the malolactic fermentation pathway of *L. lactis* (25). One of the clones contained the 5' end of a second ORF starting 15 base pairs downstream of the stop codon of *mleS*. It was suggested that this open reading frame would be *mleP* coding for the malate transporter, the second protein of the pathway. The complete second reading frame, 1,278 base pairs in length, was cloned as described under "Experimental Procedures," and the nucleotide sequence was determined. A putative Shine-Dalgarno sequence AAGG is found 16 nucleotides upstream of the start codon (ATG). The stop codon (TAA) is closely followed by an inverted repeat that has features typical of a putative rho-independent transcription termination signal (35). This organization suggests that the two genes are organized in an operon. To verify this, total RNA was isolated from *L. lactis* IL1441 grown in medium on glucose with and without additional malate. Malolactic enzyme coded by *mleS* is an inducible enzyme (18). The results demonstrated that in malate grown cells a single transcript of approximately 3 kb hybridized with a probe specific for *mleP* and with plasmid p191A containing *mleS* and a 5' fragment of *mleP*. No band was detected when malate was omitted from the growth medium (not shown). The *mleP* sequence is available under accession number X75982.

Analysis of the deduced MleP amino acid sequence reveals a hydrophobic protein of 425 amino acid residues with a predicted mass of 46.7 kDa. Screening of the available databases showed that the protein revealed homology to the citrate transporters of lactic acid bacteria (CitPs) and to the Na^+ dependent citrate carriers of *K. pneumoniae* (CitS), *Salmonella pullorum* (CitC) and *Salmonella dublin* (CitC). Like the lactococcal CitPs, the Na^+-dependent transporters form a group of proteins with almost identical primary sequences (>95%). The

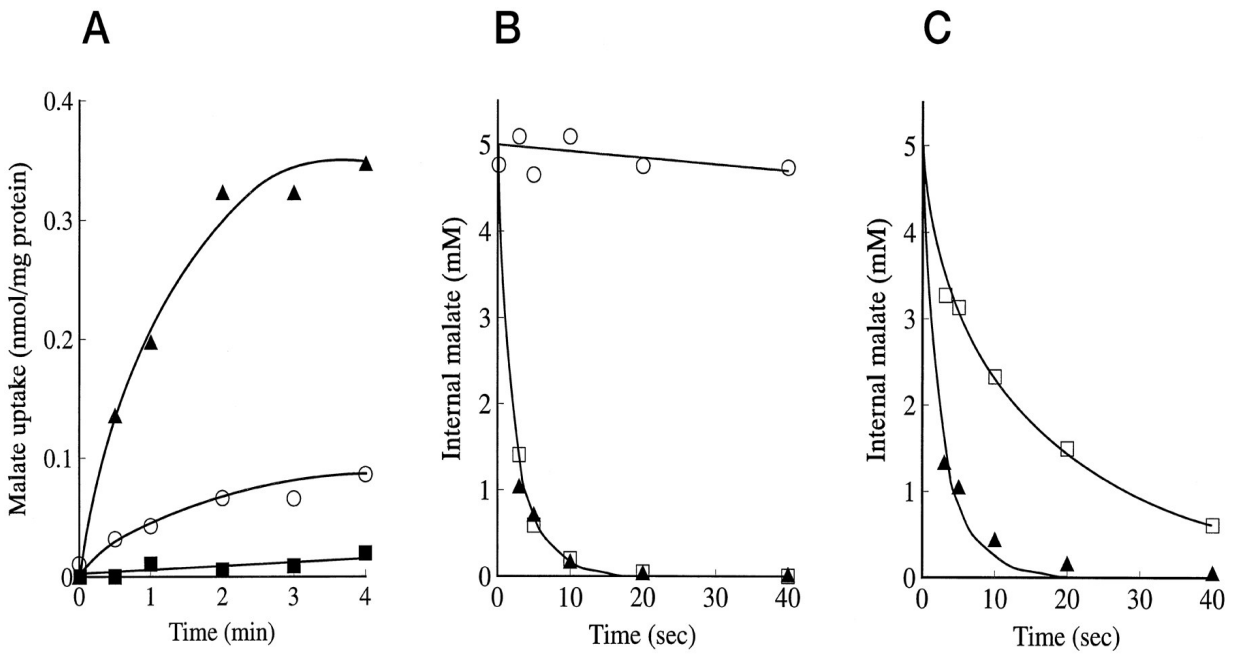

Figure 3. **Malate uptake in the presence of a PMF (A) and exchange (B,C) catalysed by MleP.** *Panel A.* Malate (13.1 μM) uptake by membrane vesicles of MG1363/pMB*mleP* fused with COVs was assayed in the presence of the electron donor system cytochrome *c*/TMPD/potassium ascorbate. (circles) no ionophores, (triangles) valinomycin and (filled squares) nigericin. *Panels B* and *C*. Membrane vesicles of *L.lactis* LL108 expressing MleP, fused with liposomes were preloaded with 5 mM L[^{14}C]malate with (C) and without (B) 100 mM KSCN and, subsequently, diluted 100-fold into buffer containing 5 mM of lactate (open squares), malate (triangles), no additions (circles). Valinomycin (*panel B*) and nigericin (*panels B and C*) were present at 1 and 0.5 μM respectively.

alignment of MleP with representative sequences from these two groups is shown in Figure 1. MleP is most similar to CitP with 48% identical residues and shares 30% sequence identity with CitS. Overall, the alignment shows 86 (19%) conserved residues with an additional 85 similar residues. A glycine rich region around residue 175 in MleP and approximately the C-terminal 60 residues are the most conserved regions in the family. Figure 2 shows the hydropathy profiles of the individual members (thin lines) and the average profile of the family (bold). The profiles are remarkably similar, indicative of the same global structure.

Functional expression of MleP. To determine whether the cloned gene identified as *mleP* is the malate transport protein involved in malolactic fermentation, the gene was expressed in *L. lactis* MG1363 and LL108, strains not able to ferment malate. Cytoplasmic membranes with a right-side-out orientation prepared from *L. lactis* MG1363 harboring pMB*mleP* (see "Experimental Procedures") were fused to proteoliposomes reconstituted with purified beef heart cytochrome *c* oxidase (COVs) as a PMF generating system. In these hybrid membranes a PMF (inside negative and alkaline relative to the outside) is generated in the presence of the electron donor system potassium ascorbate, TMPD and cytochrome *c*. In the presence of a PMF the hybrid membranes took up a low but significant amount of [^{14}C]malate (Figure 3A, circles). Control experiments with hybrid membranes prepared from membrane vesicles of strain MG1363 without pMB*mleP* showed no uptake under identical conditions (not shown). Therefore, the product of the *mleP* gene is a malate transporter. The proton motive force generated by cytochrome *c* oxidation is composed of a membrane potential ($\Delta\psi$) and a pH gradient (ΔpH). The role of each component of the PMF in driving [^{14}C]malate uptake was investigated by manipulating ΔpH and $\Delta\psi$ with the ionophores nigericin, a K^+/H^+ antiporter and valinomycin, a K^+ pore. In the presence of nigericin, when the PMF consists solely of a membrane potential, no uptake was observed indicating that the membrane potential is not a driving force for malate transport (closed squares). On the other hand, in the presence of valinomycin, when the PMF is composed solely of a pH gradient, a strong stimulation of

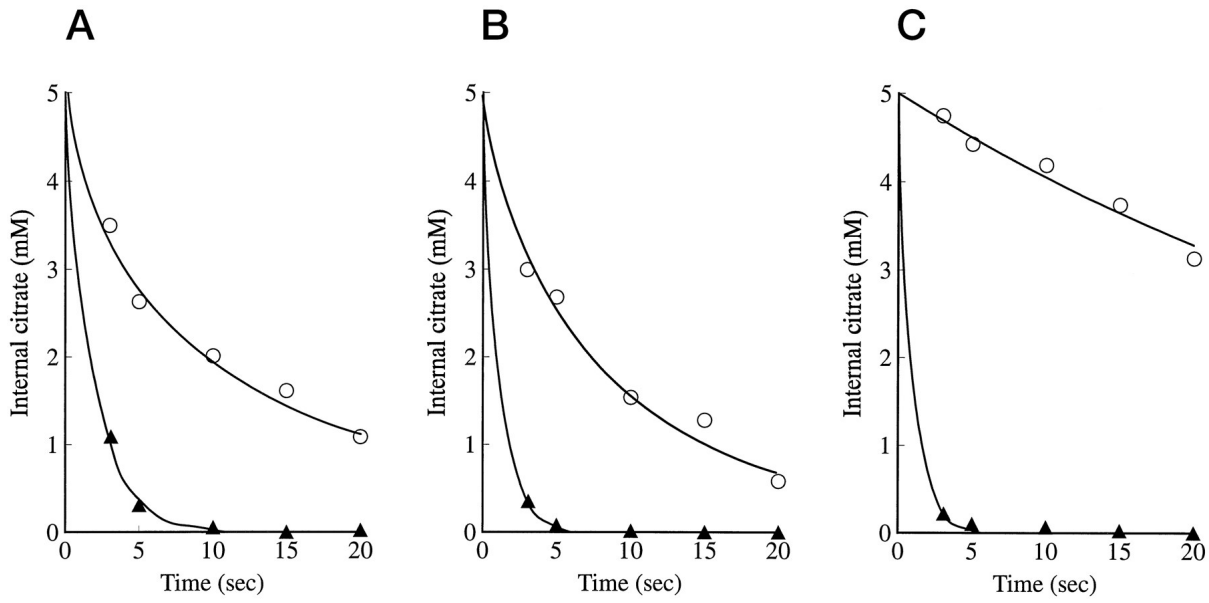

Figure 4. **Effect of the sodium ion concentration on homologous exchange and efflux catalysed by CitS.** RSO membrane vesicles of *E. coli* BL21(DE3) expressing CitS were preloaded with 5 mM [^{14}C]citrate and with no further additions (A), with 1 mM NaCl (B) and with 75 mM NaCl (C). The Cl^- concentration was kept constant by adding compensating amounts of KCl. The membranes were diluted 100-fold into buffer containing the same NaCl and KCl concentrations, in the presence (triangles) or the absence (circles) of 5 mM citrate. Valinomycin and nigericin were present at 1 and 0.5 μM, respectively.

malate uptake was observed indicating that the membrane potential counteracts malate transport (triangles) and that net negative charge is translocated across the membrane during turnover.

A second important feature of the malate carrier involved in malolactic fermentation is the physiological mode of transport, i.e. heterologous malate-lactate exchange (3). Right-side-out membrane vesicles of *L. lactis* LL108 harboring pMB*mleP* were fused with liposomes and preloaded with 5 mM L-[^{14}C]malate. 100-fold dilution of the membranes in buffer did not result in significant release of label within the first 40 s, indicating that efflux of malate down a concentration gradient is a slow process (Figure 3B, circles). In contrast, dilution of the preloaded membranes in buffer containing an equimolar concentration of unlabeled malate resulted in rapid release of internal labeled malate indicative of rapid homologous exchange (triangles). Most importantly, the same rapid release of label was observed upon dilution into buffer containing an equimolar concentration of lactate (open squares). To prevent the formation of ΔpH or $\Delta\psi$ that would counteract efflux, the ionophores valinomycin and nigericin were included in these experiments. Preloading of the membranes with the membrane permeable ion SCN^- results in the generation of a diffusion potential, negative outside, upon dilution when valinomycin is ommitted from the assay mixture. The diffusion potential significantly inhibited malate-lactate exchange whereas malate-malate exchange was not affected (Figure 3C) showing that heterologous exchange is electrogenic. The results are consistent with those found for malate transport in membrane vesicles of the wild-type malate-fermenting *L. lactis* IL1403 (3) showing that the *mleP* gene product is the malate transporter involved in malolactic fermentation.

Substrate specificity of MleP, CitP and CitS. The heterologous exchange assay demonstrated in Figure 3B provides a sensitive and unambiguous assay for the substrate specificity of a transporter in general, and was used to determine the substrate specificity of MleP and the two other representatives of the family, CitP of *Lc. mesenteroides* and CitS of *K. pneumoniae*. To use the assay it is essential that efflux is much slower than exchange. This condition is a property of precursor-product exchangers (2) and has also been demonstrated for CitP (9). In case of CitS, conditions of rapid exchange/slow efflux were sought by varying the

Table 1. **Effect of the R substituents on the ability of MleP, CitP and CitS to transport 2-hydroxycarboxylates.**

Substrate[a]	R_1	R_2	Relative rate of exchange[b] (%)		
			MleP	CitP	CitS
isocitrate	$CHCOO^-CH_2COO^-$	H	0.4 ± 0.2	2.6 ± 0.4	1.5 ± 0.7
citrate	CH_2COO^-	CH_2COO^-	0.5 ± 0.3	100 ± 8	100 ± 6
citromalate	CH_2COO^-	CH_3	6.9 ± 1.0	32 ± 1	4.6 ± 0.5
tartrate	$CHOHCOO^-$	H	18 ± 1	7.1 ± 0.2	0.9 ± 0.5
malate	CH_2COO^-	H	100 ± 8	142 ± 6	1.6 ± 0.8
2-hydroxybutyrate	CH_2CH_3	H	15 ± 3	54 ± 1	1.3 ± 0.4
2-hydroxyisobutyrate	CH_3	CH_3	16 ± 3	100 ± 1	1.9 ± 0.7
lactate	CH_3	H	91 ± 14	36 ± 1	1.7 ± 0.5
glycolate	H	H	26 ± 8	3.3 ± 0.5	1.7 ± 0.8
none	–	–	0.5 ± 0.3	1.0 ± 0.5	1.0 ± 0.6

[a]Substrates were added at a concentration of 5 mM. Equal mixtures of the L- and D- isomers were used. [b]Rates are relative to the rate observed for homologous exchange which was set at 100 %. These rates varied per vesicle preparation between 2.7 and 4.8 , 0.7 and 0.8 and 2.3 and 2.6 mM/sec for MleP, CitP and CitS, respectively. Rates are given as the average of two to four independent measurement and the standard deviation.

concentration of the symported Na^+ ion (Figure 4). With no additional Na^+ added, efflux and exchange in right-side-out membrane vesicles of *E. coli* BL21(DE3) harboring plasmid pSKΔl*citS* (17) was observed at comparable rates. Since no effort was made to work "sodium free" the observed activities are most likely due to sodium ion contaminations in the buffer (15). Addition of 1 mM Na^+ increased both the rate of efflux and exchange. However, at 75 mM added Na^+ the rate of exchange increased further, but the rate of efflux decreased. This behaviour is typical for a solute/co-ion symporter (36). The latter condition was used for the substrate specificity assay.

From previous studies it was known that MleP transports both malate and lactate, CitP transports citrate, lactate and malate (9) while for CitS no substrate other than citrate has been reported. These substrates all share the 2-hydroxylcarboxylate motive, $HO\text{-}CR_2\text{-}COO^-$. The effect of the R groups, the hydroxyl group and the carboxylate group on the ability of the three transporters to translocate the substrates was investigated subsequently.

The R substituents. Nine different 2-hydroxycarboxylates with R substituents which differ both in size and polarity were included in the assay. The results are summarized in Table 1. The main conclusion is that CitS is very specific, transporting only citrate and to a low extent citramalate, whereas MleP and CitP transport a wide variety of these substrates. MleP has a preference for the smaller substrates while CitP seems to prefer the larger molecules. In contrast to CitP, MleP does not transport citrate and only poorly citromalate, whereas CitP even transports isocitrate, which has the largest substituent, at a low but significant rate. At the other end of the spectrum, glycolate is a good substrate of MleP and a poor substrate of CitP. No clear discrimination is evident between substituents with a polar and a hydrophobic character.

The hydroxyl group. A set of compounds were selected in which the hydroxyl group of one of the transported substrates listed in Table 1 was replaced by another substituent. In line with the high specificity of CitS reported above, none of these compounds was transported by CitS (Table 2). Substrates in which the hydroxyl group is replaced by a hydrogen atom are not transported. Similarly, the hydroxyl cannot be replaced by an amino group. Replacement of the hydroxyl group by a keto group resulted in significant transport in the case of oxaloacetate, especially by MleP. This transporter could also transport glyoxylate, the 2-oxo analogue of glycolate, at a significant rate while the rate catalyzed by CitP was very low but significant.

Table 2. **Effect of OH substituents on the ability of MleP, CitP and CitS to transport a substrate.**

Substrate	OH substituent	Relative rate of exchange (%)		
		MleP	CitP	CitS
citrate	OH	0.5 ± 0.3	100 ± 8	100 ± 6
tricarballate	H	1.0 ± 0.2	2.2 ± 0.7	1.0 ±0.1
malate	OH	100 ± 8	142 ± 6	1.6 ± 0.8
succinate	H	0.4 ± 0.1	0.5 ± 0.3	1.0 ± 0.5
oxaloacetate	O	27 ± 4	14 ± 1	1.5 ± 0.8
aspartate	NH_3^+	0.7 ± 0.3	1.3 ± 0.3	0.8 ± 0.5
lactate	OH	91 ± 14	36 ± 1	1.7 ± 0.5
propionate	H	0.5 ± 0.1	0.7 ± 0.3	1.3 ± 0.4
pyruvate	O	1.2 ± 0.3	4.2 ± 0.7	1.9 ± 0.8
alanine	NH_3^+	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.4
glycolate	OH	26 ± 8	3.3 ± 0.5	1.7 ± 0.8
acetate	H	0.5 ± 0.3	0.3 ± 0.2	1.0 ± 0.5
glyoxylate	O	13 ± 2	2.2 ± 0.2	0.9 ± 0.5
glycine	NH_3^+	0.7 ± 0.3	1.1 ± 0.3	0.9 ± 0.5
2-hydroxybutyrate	OH on C2	15 ± 3	54 ± 1	1.3 ± 0.4
3-hydroxybutyrate	OH on C3	1.1 ± 0.5	3.9 ± 0.2	1.5 ± 0.6
none	-	0.5 ± 0.3	1.0 ± 0.5	1.0 ± 0.6

[a]Details are in the legend to Table 1.

The latter was also observed for pyruvate, the 2-oxocarboxylate analogue of lactate, for both MleP and CitP. The position of the hydroxyl group relative to the caboxylate group was investigated by comparing 2-hydroxybutyrate and 3-hydroxybutyrate. A low but significant transport activity with the latter substrate was observed with CitP but not with MleP. In conclusion, MleP and CitP have the highest activity with 2-hydroxycarboxylates but significant activity is observed with some 2-oxocarboxylates and in case of CitP even a 3-hydroxycarboxylate.

The carboxylate group. Three different analogs of lactate with different substituents replacing the carboxylate group were tested in the exchange assay (Table 3). A common feature of the analogs 1,2-propanediol, methyllactate and glyceraldehyde is that the charge of the carboxylate is removed. In addition to the aldehyde group replacing the carboxylate, glyceraldehyde has a hydroxyl group at the C3 position. This might have an additional effect on the suitability as a substrate but, on the other hand, tartrate, an analogue of malate, has the same feature and is transported both by MleP and CitP (Table 1). None of the analogs was transported by any of the transporters emphasizing the relevance of the carboxylate group in the motif.

DISCUSSION

Secondary PMF-generating pathways were discovered only in the last decade. Oxalate decarboxylation in *Oxalobacter formigenes* and malolactic fermentation in *L. lactis* were the first systems described in detail (3, 6). Both pathways consist of only two enzymes, a secondary transporter and a cytoplasmic decarboxylase. The secondary transporters that take up the substrate in exchange for the decarboxylation product (precursor-product exchange), and thereby generate the membrane potential, play a central role in the pathways. Recently, the structural gene coding for the oxalate-formate exchanger OxlT of *O. formigenes* was cloned and sequenced (37). Here, we report the cloning and sequencing of MleP, the malate-lactate exchanger of *L. lactis*. The cloned gene was expressed in the malolactic fermentation-negative *L. lactis* strains MG1363 and LL108. Functional characterization in membrane vesicles derived

Table 3. **Effect of COO^- substituents on the ability of MleP, CitP and CitS to transport a substrate.**[a]

Substrate	COO^- substituent	Relative rate of exchange (%)		
		MleP	CitP	CitS
lactate	COO^-	91 ± 14	36 ± 1	1.7 ± 0.5
methyl-L-lactate	$COOCH_3$	1.0 ± 0.1	0.8 ± 0.1	1.3 ± 0.4
1,2-propanediol	CH_2OH	0.4 ± 0.3	0.4 ± 0.2	1.3 ± 0.4
glyceraldehyde	CHO	0.9 ± 0.5	0.7 ± 0.5	1.1 ± 0.5
none	–	0.5 ± 0.3	1.0 ± 0.5	1.0 ± 0.6

[a]Details are in the legend to Table 1.

from these cells showed that the gene product conferred the same transport characteristics as was observed before in membrane vesicles of the malate fermenting wild type strain IL1403 (3). The cloned transporter catalyzed efficient heterologous malate-lactate exchange, and unidirectional uptake into the membranes was counteracted by the membrane potential.

The *mleP* gene of *L. lactis* is not homologous to the *oxlT* gene of *O. formigenes* indicating that genes coding for membrane potential-generating secondary transporters do not form a separate gene family. MleP was found to be homologous to the membrane potential-generating citrate transporter CitP of lactic acid bacteria and the Na^+ dependent citrate transporters CitS (13) and CitC (38) of *K. pneumoniae* and *Salmonella* species. The homology to CitS and CitC which are metabolic energy-dissipating transporters suggests that MleP and CitP, and membrane potential generating secondary transporters in general are conventional secondary transporters. The membrane topology of CitS was recently reported to be quite different from the 12 transmembrane helix motif usually observed for secondary transporters (17). CitS traverses the membrane 9 times (the bars in Figure 1) with a cytoplasmic amino terminus and a periplasmic carboxyl terminus. The sequence homology and the highly conserved hydropathy profile of the members in the family strongly suggest that MleP and CitP fold in a similar fashion in the membrane. In this structural model the two most conserved regions in the alignment, shown in Figure 1, are located in the periplasmic loop between helices V and VI and the cytoplasmic loop preceding the C-terminal helix IX. The alignment shows 6 conserved positively charged amino acid residues of which only Arg407 (MleP numbering) is predicted to be located in the membrane, in putative helix IX. Since MleP, CitP and CitS transport negatively charged substrates this Arg residue could play a role in substrate binding and/or transport.

Previous studies had shown that MleP, CitP and CitS transport one or more of the structurally related substrates citrate, malate and lactate (3, 9, 14), and it was noted that these substrates all contain the motif $HO\text{-}CR_2\text{-}COO^-$ (9) . In the present study the importance of the hydroxyl and carboxylate groups of the substrates was investigated. None of a limited number of lactate analogs in which the carboxylate group was methylated or replaced by an hydroxyl or aldehyde group could be translocated by any of the transporters. This suggests that the carboxylate and possibly the negative charge of this group is essential. A larger number of analogs showed that replacement of the hydroxyl group by a hydrogen atom or an amino group completely abolished transport activity (Table 2). However, replacement by a keto group resulted to some extend in activity with MleP and CitP, especially with oxaloacetate. Moving the hydroxyl to the C3 position resulted in a low transport activity by CitP. Possibly, hydrogen bonding between a residue on the transporter and the hydroxyl or keto group on the substrates is essential for translocation. The transport activity of CitP with oxaloacetate is remarkable since oxaloacetate is the first metabolic intermediate in the citrate degradation pathway in

lactic acid bacteria (39). Since the 2-hydroxycarboxylates are the physiological and preferred substrates of these carriers we have termed the family the 2-hydroxycarboxylate transporter family.

A typical feature of membrane potential-generating secondary transporters like MleP and CitP is the ability to translocate two structurally related substrates, i.e. malate/lactate and citrate/lactate, respectively. The transporters specifically recognize the common 2-hydroxycarboxylate motif. At the same time, MleP and CitP need to be quite tolerant towards the R_1 and R_2 groups since the cytoplasmic conversion of the substrate into the product not only results in a smaller molecule but also removes the charge on one of the R groups. Charge removal is crucial and results in the generation of the membrane potential. A similar tolerance towards the R groups is not a physiological requirement for the Na^+-dependent citrate transporter CitS that functions as a Na^+/H^+ symporter (14-16). Consistent with this observation, the results in Table 1 show that CitS has a very narrow substrate specificity (mainly citrate), while MleP and CitP transport a wide range of 2-hydroxycarboxylates with different R substituents. Remarkably, the R groups of the transported substrates range in size from the smallest possible, *i.e.* hydrogen atoms in glycolate, to the R groups of the physiological substrates, *i.e.* malate for MleP and citrate for CitP. Larger R groups as in citramalate for MleP and isocitrate for CitP result in very poor translocation. Apparently, the translocation site is optimized for the physiological substrates and smaller R groups are compensated for by the conformation of the protein or a varying amount of cotransported water (40, 41). In the case of CitS smaller R groups reduce transport drastically, possibly because the R groups are essential for Na^+ binding.

The members of the 2-hydroxycarboxylate transporter family are a potent experimental system to study the relation between the primary sequence and substrate specificity. Currently, we are dissecting the binding and translocation events kinetically, analysing the stereoselectivity of the transporters and identifying residues in the primary sequences that are located in the binding pockets of MleP, CitP and CitS. Such studies will eventually give a detailed model of the binding sites and explain how the details in similar structures result in important functional differences.

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REFERENCES

1. Konings , W.N., Lolkema, J.S. and Poolman, B. (1995) *Arch. Microbiol*. **164**, 235-242.
2. Lolkema, J.S., Poolman, B. and Konings, W.N. (1996) In: Handbook of Biological Physics 2 (Ed. Konings, W.N., Kaback, H.R. and Lolkema, J.S.) Chapter 11, p229-260. Elsevier, Amsterdam.
3. Poolman, B., Molenaar, D., Smid, E.J., Ubbink, T., Abee, T., Renault, P.P. and Konings, W.N. (1991) *J. Bacteriol*. **173**, 6030-6037.
4. Salema, M., Poolman, B., Lolkema, J.S., Loureiro Dias, M.C. and Konings, W.N. (1994) *Eur. J. Biochem*. **225**, 289-295.
5. Olsen, E.B., Russel, J.B. and Henick-Kling, T. (1991) *J. Bacteriol*. **173**, 6199-6206.
6. Anantharam, V., Allison, M.J. and Maloney, P.C. (1989) *J. Biol. Chem*. **264**, 7244-7250.
7. Molenaar, D., Bosscher, J.S., Ten Brink, B., Driessen, A.J.M. and Konings, W.N. (1993) *J. Bacteriol*. **175**, 2864-2870.
8. Abe, K., Hayashi, H. and Maloney, P.C. (1996) *J. Biol. Chem.* **271**, 3079-3084.
9. Marty-Teysset, C., Lolkema, J.S., Schmitt, P., Divies, C. and Konings, W.N. (1995) *J. Biol. Chem*. **270**, 25370-25376.
10. Marty-Teysset, C., Posthuma, C., Lolkema, J.S., Schmitt, P., Divies, C. and Konings, W.N. (1996) *J. Bacteriol*. **178**, 2178-2185.
11. David, S., van der Rest, M.E., Driessen, A.J.M., Simons, G. and de Vos, W.M. (1990) *J. Bacteriol*. **172**, 5789-5794.

12. Vaughan, E.E., David, S., Harrington, A., Daly, C., Fitzgerald, G.F. and de Vos, W.M. (1996) *Appl. Environ. Microbiol.* **61**, 3172-3176.
13. van der Rest, M.E., Siewe, R.M., Abee, T., Schwarz, E., Oesterhelt, D. and Konings, W.N. (1992) *J. Biol. Chem.* **267,** 8971-8976.
14. van der Rest, M.E., Molenaar, D. and Konings, W.N. (1992) *J. Bacteriol*. **174**, 4893-4898.
15. Lolkema, J.S., Enequist, H. and van der Rest, M.E. (1994) *Eur. J. Biochem*. **220**, 469-475.
16. Pos, K.M. and Dimroth, P. (1996) *Biochemistry* **35**, 1018-1026.
17. van Geest, M. and Lolkema, J.S. (1996) *J. Biol. Chem*. **271**, 25582-25589.
18. Renault, P.P. and Heslot, H. (1987) *Appl. Environ. Microbiol.* **53**, 320-324.
19. Leenhouts, K., Buist, G., Bolhuis, A., ten Berge, A., Kiel, J., Mirau, I., Dabrowska, M., Venema, G. and Kok, J. (1996) *Mol. Gen. Genet.* **253**, 217-224.
20. De Man, J. C., Rogosa, M. and Sharp, M.E. (1960) *J. Appl. Bacteriol.* **23**, 130-135.
21. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring harbor, NY.
22. Simon, D., Rouault, A. and Chopin, M.C. (1985) *FEMS Microbiol. Lett.* **26**, 239-241
23. Leenhouts, K.J., Kok, J. and Venema, G. (1990) *Appl. Env. Microbiol*. **56**, 2726-2735.
24. Holo, H. and Nes, I.F. (1989) *Appl. Env. Microbiol*. **55**, 3119-3123.
25. Ansanay, V., Dequin, S., Blondin, B. and Barre, P. (1993) *FEBS Lett.* **332**, 74-80.
26. Yanish-Perron, C., Vieira, J. and Messing, J. (1985) *Gene* **33**, 103-119.
27. Lopez de Felipe, F., Magni, C., de Mendoza, D. and Lopez, P. (1995) *Mol. Gen. Genet*. **246**, 590-599.
28. Kok, J., Vossen, J.M.B.M. and Venema, G. (1984) *Appl. Env. Microbiol*. **48**, 726-731.
29. Otto, R., Lageveen, R.G., Veldkamp, H. and Konings, W.N. (1982) *J. Bacteriol*. **149**, 733-738.
30. Driessen, A.J.M., de Vrij, W. and Konings, W.N. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7555-7559.
31. Driessen, A.J.M., Brundage, L., Hendrick, J.P., Schiebel, E. and Wickner, W. (1991) *Methods Cell Biol*. **34**, 147-165.
32. Mayer, D.L., Hope, M.J. and Cullis, P.R. (1986) *Biochim. Biophys. Acta* **858**, 161-168.
33. Kaback, H.R. (1971) *Methods Enzymol.* **22**, 99-120
34. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem*. **193**, 265-275.
35. De Vos, W. M. (1987) *FEMS Microbiol. Rev.* **46**, 281-295.
36. Viitanen, P.V., Garcia, M.L., Foster, D., Kaczorowski, G.J. and Kaback, H.R. (1983) *Biochemistry* **22**, 2531-2536.
37. Abe, K., Ruan, Z-S. and Maloney, P. C. (1996) *J. Biol. Chem.* **271**, 6789-6793.
38. Ishiguro, N., Izawa, H., Shinagawa, M., Shimamoto, T. and Tsuchiya, T. (1992) *J. Biol. Chem.* **267**, 9559-9564.
39. Marty-Teysset, C., Lolkema, J. S., Schmitt, P., Divies, C. and Konings, W. N. (1996) *J. Bact.* **178**, 6209-6215.
40. Loo, D.D.F., Zeuthen, T., Chandy, G. and Wright, E.M. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 13367-13370.
41. Zuethen, T. (1995) *Int. Rev. Cytol.* **160**, 99-161.
42. Eisenberg, D. (1984) *Ann. Rev. Biochem.* **53**, 595-623.

Stereoselectivity of the Membrane Potential Generating Citrate (CitP) and Malate (MleP) Transporters of Lactic Acid Bacteria.

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SUMMARY

The citrate transporter of *Leuconostoc mesenteroides* (CitP) and the malate transporter of *Lactococcus lactis* (MleP) are homologous proteins that catalyze citrate-lactate and malate-lactate exchange, respectively. Both transporters transport a range of substrates that contain the 2-hydroxycarboxylate motif, HO-CR_2-COO^- (6). In this study we have analyzed binding and translocation properties of CitP and MleP for a wide variety of substrates and substrate analogs. Modification of the OH or the COO^- groups of the 2-hydroxycarboxylate motif drastically reduced the affinity of the transporters for the substrates, indicating their relevance in substrate recognition. Both CitP and MleP were strictly stereoselective when the R group contained a second carboxylate group; the S-enantiomers were efficiently bound and translocated, while the transporters had no affinity for the R-enantiomers. The affinity of the S-enantiomers, and of citrate, was at least one order of magnitude higher than for lactate and other substrates with uncharged R groups, indicating a specific interaction between the second carboxylate group and the protein that is responsible for high affinity binding. MleP was not stereoselective in binding when the R groups are hydrophobic and as large as a benzyl group. However, only the S-enantiomers were translocated by MleP. CitP had a strong preference for binding and translocating the R-enantiomers of substrates with large hydrophobic R groups. These differences between CitP and MleP explain why citrate is a substrate of CitP and not of MleP. The results are discussed in the context of a model for the interaction between sites on the protein and functional groups on the substrates in the binding pockets of the two proteins.

INTRODUCTION

In the last decade a growing number of secondary transporters have been discovered that generate rather than consume metabolic energy (1). These transporters have been termed precursor-product exchangers since they catalyze the coupled uptake of a substrate into the cell and exit of a metabolic end product into the medium. Well studied examples are the oxalate transporter (OxlT) of *Oxalobacter formigenes* (2-4) and the citrate (CitP) and malate (MleP) transporters of the lactic acid bacteria *Leuconostoc mesenteroides* (5, 6) and *Lactococcus lactis* (6, 7), respectively. CitP exchanges divalent citrate and MleP divalent malate for monovalent lactate, an end product of both citrate and malate degradation (7, 8). The net charge movement during this exchange results in a membrane potential of physiological polarity. Furthermore, decarboxylation reactions in the breakdown of citrate and malate consume scalar protons and thus generate a pH gradient of physiological polarity. The result of the combined activities of precursor-product exchange and decarboxylation is a proton motive force that is sufficiently high to drive ATP-synthesis via F_0F_1-ATPase.

Citrate transporters (CitPs) from three different lactic acid bacteria have been cloned and sequenced and the translated amino acid sequences were found to be 98% identical (9-11). Also, functionally the transporters were indistinguishably (11). More recently, the malate transporter MleP of *L. lactis* was cloned and sequenced and found to be homologous to CitP,

sharing 48% identical residues. Both transporters belong to the bacterial 2-hydroxycarboxylate transporter (2HCT) family that contains both membrane potential-generating and -dissipating members. A well studied transporter of the latter group is the sodium ion motive force-driven citrate transporter of *Klebsiella pneumoniae,* CitS (12-16). Despite the different energetics, the homology between the proteins strongly suggests that the membrane potential generating transporters are "classical" secondary transporters.

A study of the substrate specificity of CitP, MleP and CitS revealed that all three transporters specifically transport substrates containing a 2-hydroxycarboxylate ($HO\text{-}CR_2\text{-}COO^-$) motif. The transporters were found to differ in their tolerance towards the two R substituents of the substrates (6). The Na^+ motive force driven CitS was found to have a very narrow specificity, transporting mainly citrate. In contrast, the precursor-product exchangers MleP and CitP were found to have broad and overlapping specificities, the main difference being that CitP transports larger molecules than MleP. The largest substrate accepted by MleP is malate whereas CitP, in addition to malate, also transports citrate. Both transporters translocate glycolate, the smallest 2-hydroxycarboxylate with the R-groups representing H-atoms. The ability to accept various R substituents that differ in size, and most importantly in charge, is essential for the function of CitP and MleP, since they catalyze exchange between a divalent precursor (i.e. citrate or malate) and a monovalent product (i.e. lactate).

In this study we elaborate on the binding and catalytic properties of CitP and MleP for the substrates and a number of substrate analogs to determine the stereoselectivity of the transporters and, thus, gain insight in the nature of the substrate binding pockets of these proteins. The resulting models for substrate binding in CitP and MleP are similar and provide the basis for the physiological function of the transporters. Interactions with the OH and COO^- groups of the 2-hydroxycarboxylate motif are essential for recognition of the substrates by the transporters. In addition, a localised electrostatic interaction with the second carboxylate of the precursor molecules citrate and malate is responsible for high-affinity binding relative to the affinity for the product lactate. Steric restrictions during translocation in MleP are responsible for the inability of MleP to transport the larger citrate molecule.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions. *Lactococcus lactis* MG1363 is a malate and citrate fermentation negative strain that does not contain endogenous citrate and malate transport systems. *L. lactis* MG1363 was transformed with the *E. coli/L. lactis* shuttle vectors pMB-citP or pMB-mleP (6) to express the citrate transporter CitP and the malate transporter MleP, respectively. In the vectors pMB-mleP and pMB-citP the *mleP* gene from *L. lactis* IL1403 (6) and the *citP* gene from *L. mesenteroides* ssp. *mesenteroides* (11) were cloned downstream of the constitutive promotor of the *citP* gene cluster of *L. lactis* NCDO176 (17). The cells were grown in M17 broth (Difco) supplemented with 0.5% (w/v) glucose and 5 μg/mL erythromycin. The cells were grown at 30 °C in closed serum bottles without shaking.

Preparation of membrane vesicles. Cells of *L. lactis* MG1363 expressing either MleP or CitP were harvested at the end of the exponential growth phase at an optical density of 0.8 measured at 660 nm (OD_{660}), washed with 50 mM potassium phosphate (pH 7), resuspended in the same buffer at an OD_{660} of 500, and rapidly frozen in liquid nitrogen until use. Right-side-out membrane vesicles were prepared by the osmotic shock lysis procedure essentially as described by Otto *et al*. (18). (S)-malate (L-malate) was present at a concentration of 5 mM throughout the procedure to load the vesicles with (S)-malate. The vesicles were rapidly frozen in liquid nitrogen in 50 mM potassium phosphate (pH 6), containing 5 mM (S)-malate. The protein concentration was determined as described by Lowry *et al*. (19).

Exchange and counterflow in membrane vesicles. Membrane vesicles of *L. lactis* MG1363 containing either CitP or MleP were washed in 50 mM potassium phosphate (pH 6), containing 5 mM (S)-malate and concentrated by centrifugation for 15 min in an Eppendorf centrifuge operated at full speed, followed by resuspension in the same buffer. For exchange measurements, the internal pool of (S)-malate was labeled with (S)-[^{14}C]malate by incubating the concentrated membranes with 186.7 μM L-[1,4(2,3)^{14}C]malate for 1 h at room temperature in the presence of 100 μM valinomycin and 50 μM nigericin. Aliquots of 2 μL were diluted into 200 μL of the potassium phosphate buffer containing various substrates at the indicated concentrations at 20 °C. For counterflow experiments, the (S)-malate-loaded concentrated vesicles were diluted 100-fold into 200 μl buffer containing 9.8 μM L-[1,4(2,3)^{14}C]malate and different concentrations of various substrates when indicated. Valinomycin and nigericin were present at 1 and 0.5 μM, respectively. Final membrane protein concentrations in the assays were

between 250 and 350 μg/mL. Reactions were stopped at the indicated times by addition of 4 mL ice-cold 0.1 M LiCl and rapid filtration over 0.45 μm pore size cellulose nitrate filters (Schleicher & Schuell). The filters were rinsed once with 4 mL of ice-cold 0.1 M LiCl and transferred to scintillation vials to determine the internal radioactivity.

Evaluation of the data. Initial rates of exchange were determined by fitting the data to an exponential decay as described previously (6) using nonlinear fitting procedures provided by the Sigma Plot software (Jandel Scientific, San Rafael, CA). The affinity constant for a substrate in the external buffer and the maximal rate of heterologous exchange between the substrate and internal (S)-malate were determined by measuring the initial rates of exchange at different concentrations of the substrate in the external buffer. The data were fitted to an equation describing competitive inhibition in which [S] is the concentration of the substrate in the dilution buffer, [I] the concentration of (S)-[^{14}C]malate in the external buffer caused by the dilution of the (S)-malate loaded vesicles (i.e. routinely 50 μM), and K_i the affinity constant for (S)-malate determined from homologous (S)-malate exchange.

Equation 1 $$v = V_{max} \frac{[S]}{[S] + Km\,(1+ [I]/K_i)}$$

Initial rates of counterflow were estimated from internalized label measured at the 3 and 5 sec time points that were measured in triplicate. Data was used only when the uptake increased proportional with time to ensure initial rate conditions. The level of inhibition of (S)-malate counterflow by a substrate was measured at different concentrations of the substrate in the external buffer. The inhibition constant for the substrate was estimated by fitting the data to equation 1 in which [S] and K_m are concentration and affinity constant for external (S)-malate, respectively, and [I] and K_i the concentration and affinity constants for the added substrate, respectively.

Chemicals. L-[1,4(2,3)^{14}C]malate (51 mCi/mmol) was obtained from Amersham International (Buckinghamshire, UK). All other compounds were obtained from Fluca (Buchs, Switzerland) or Sigma (St. Louis, MO).

RESULTS

Exchange of (S)- and (R)-malate catalyzed by MleP. Heterologous exchange provides a sensitive and unambiguous assay for transport of a compound by a secondary transporter. The assay measures the potency of a compound in inducing efflux of a radiolabeled substrate of the transporter from preloaded membrane vesicles. The assay depends on the condition that efflux down a concentration gradient is much slower than exchange with an external substrate.

Right-side-out membrane vesicles prepared from *L. lactis* cells in which the malate transporter MleP was expressed were loaded with 5 mM (S)-[^{14}C]malate. Release of the label was very slow when diluted 100 fold into buffer, indicating that efflux of malate down a

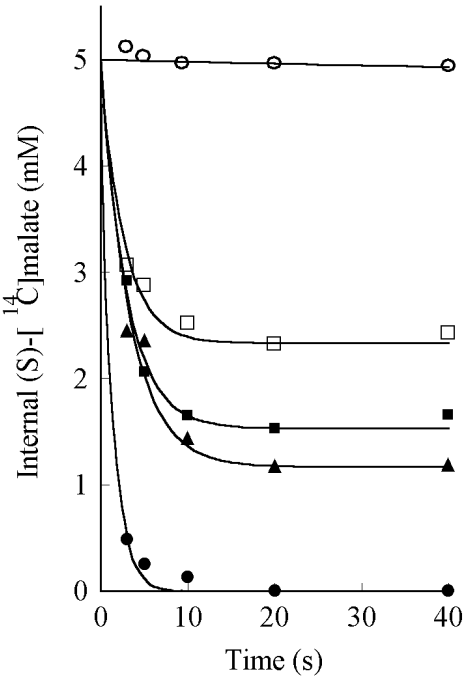
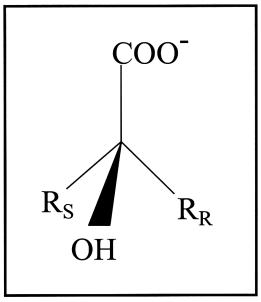


Figure 1. **(S)-Malate-(S)-malate and (R)-malate-(S)-malate exchange catalyzed by MleP.** RSO membrane vesicles of *L. lactis* MG1363 expressing MleP were preloaded with 5 mM (S)-[^{14}C]malate. The membranes were diluted 100-fold into buffer containing no further additions (open circles), 5 mM (S)-malate (closed circles), 0.1 mM (S)-malate (closed squares), 0.05 mM (S)-malate (open squares) and 5 mM (R)-malate (triangles).



	R_S	R_R
(S)-2-hydroxyglutarate	$-CH_2-CH_2-COO^-$	$-H$
(S)-malate	$-CH_2-COO^-$	$-H$
(S,S)-tartarate	$-CH(OH)-COO^-$	$-H$
(S)-mandelate	$-C_6H_5$	$-H$
(S)-2-hydroxyisovalerate	$-CH(CH_3)_2$	$-H$
(S)-lactate	$-CH_3$	$-H$
(S)-citramalate	$-CH_2-COO^-$	$-CH_3$
citrate	$-CH_2-COO^-$	$-CH_2-COO^-$
2-hydroxyisobutyrate	$-CH_3$	$-CH_3$
glycolate	$-H$	$-H$

Figure 2. **Stereochemistry of 2-hydroxycarboxylates.** Throughout this paper, the position of the two R side chains of the 2-hydroxy-carboxylate motif HO-CR_2-COO^- in the binding pocket will be indicated as R_S and R_R (inset). By definition, the R_S and R_R position correspond to the side occupied by the R group in the S- and R-enantiomeric forms of monosubstituted 2-hydroxycarboxyl-ates (HO-CHR-COO^-), respectively. The S-enantiomers of the substrates used in this study are indicated in the table on the right. The corresponding R-enantiomers can be visualized by interchanging the R_R and R_S substituents.

concentration gradient is a slow process (Figure 1, open circles). In contrast, equilibrium exchange which results from dilution into buffer containing 5 mM unlabeled (S)-malate was very rapid (closed circels). At lower external (S)-malate concentrations of 50 (open squares) and 100 (closed squares) μM the rate of exchange was slower. Also, the total amount of label released from the vesicles at equilibrium decreases with decreasing external substrate concentrations since this depends on the ratio of labeled and unlabeled exchangeable substrates at both sides of the membrane. When the 5 mM unlabeled (S)-malate in the external buffer was replaced by the same concentration of the stereoisomer (R)-malate, a rapid release to about 30 % of the internal label was observed (Figure 1, triangle). This amount of released label was lower than expected and indicates an external substrate concentration of just above 100 μM [compare to the level obtained with 100 μM (S)-malate]. Therefore, the observed exchange is not caused by (R)-malate but by a transportable contamination present at about 2% in (R)-malate (100 μM/5 mM), most likely (S)-malate. The extent of heterologous exchange with (R)-malate can be estimated from the second phase of the curve and is undetectable in this experiment (triangles). In conclusion, MleP is highly selective for the S-enantiomer of malate. The presence of the contamination in (R)-malate limits the kinetic analysis of this substrate. A similar contamination of substrates of either MleP or CitP was found to be present in (S)-citramalate.

Stereoselectivity in transport catalyzed by CitP and MleP. Heterologous exchange rates of (S)-malate and the R- and S-enantiomers of a number of monosubstituted 2-hydroxycarboxylates, i.e., HO-CHR-COO^-, were determined for both MleP and CitP. The R substituents were either hydrophilic, i.e., malate, tartrate and 2-hydroxyglutarate, or hydrophobic, i.e., lactate, 2-hydroxyisovalerate and mandelate (see Figure 2).

For CitP, the substrates with hydrophilic/charged R groups yielded much higher exchange rates when in the S-enantiomeric than in the R-enantiomeric form (Figure 3A). Relative to homologous (S)-malate exchange, heterologous exchange with (S)-2-hydroxyglutarate and (S)-tartrate was roughly 5 times slower under the conditions of the experiments. Remarkably, CitP showed a preference for the R-enantiomer of the substrates with the hydrophobic substituents. The stereoselectivity with the hydrophobic R-groups was less stringent than with the hydrophilic R-groups. (S)-Citramalate is like a "hybrid" of (S)-malate and (R)-lactate with a hydrophilic CH_2COO^- group at the R_S position and a hydrophobic CH_3 group at the R_R position (see Figure 2). As expected, (S)-citramalate was preferred over (R)-citramalate, but the exchange rate with (S)-citramalate was low compared to (S)-malate.

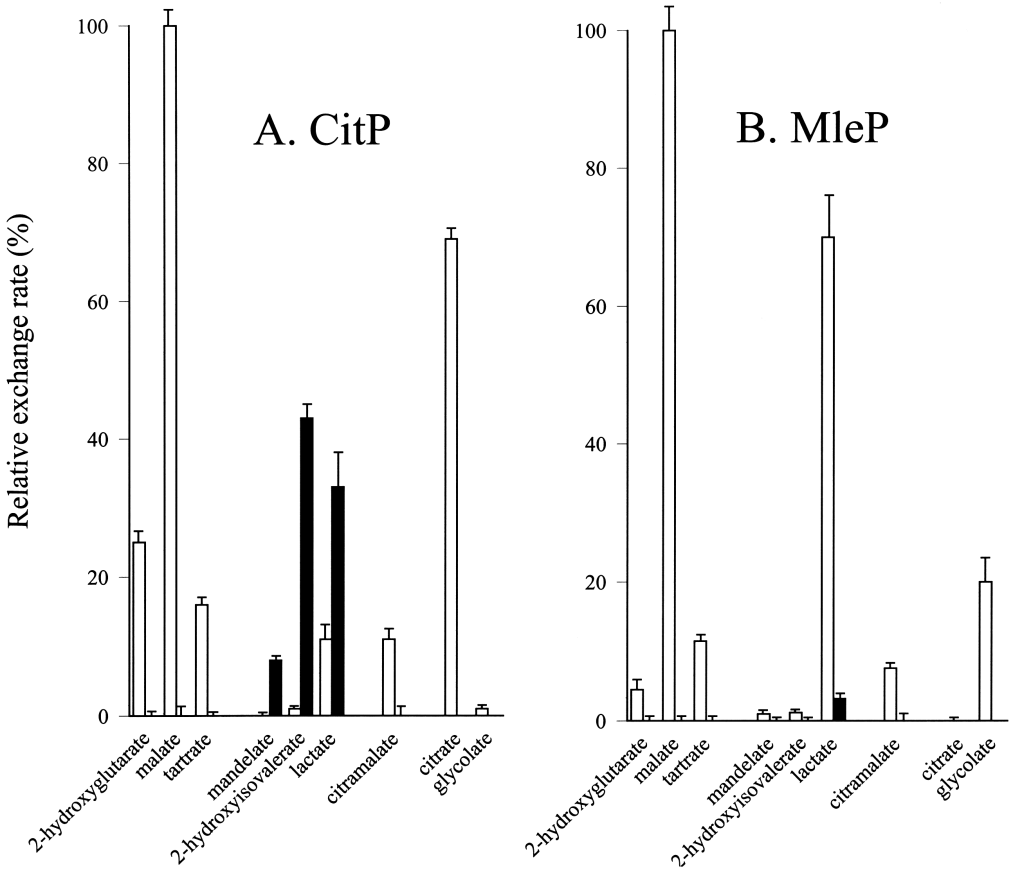


Figure 3. **Heterologous exchange rates catalyzed by CitP (A) and MleP (B).** RSO membrane vesicles of *L. lactis* MG1363 expressing CitP (A) and MleP (B) were preloaded with 5 mM (S)-[^{14}C]malate and diluted 100-fold into buffer containing the indicated 2-hydroxycarboxylates at 5 mM. When appropriate, the left bars (open) and the right bars (black) correspond to the S- and R-enantiomers of the substrates, respectively. Rates are given relative to the rate observed for homologous (S)-malate exchange that was set at 100%. The rates for homologous (S)-malate exchange varied per vesicle preparation between 1 and 1.2 mM/sec for CitP and between 3 and 3.4 for MleP. Indicated rates and error bars represent average values of 2-4 independent measurements and the standard deviations, respectively.

MleP, like CitP, preferred the S-enantiomers of substrates bearing hydrophilic substituents and catalyzed homologous exchange of (S)-malate considerably faster than heterologous exchange with (S)-2-hydroxyglutarate and (S)-tartrate (Figure 3B). MleP clearly differed from CitP in that, in case of substrates containing a hydrophobic substituent, it preferred to transport the S-enantiomers as well. The preference was most clear for lactate. The substrates mandelate and 2-hydroxyisovalerate, which have larger hydrophobic substituents, result in low exchange rates, but also for these substrates the preference for the S-enantiomer was significant. In fact, no exchange with the R-enantiomers was observed even at concentrations as high as 20 mM and when assayed for longer periods of times (data not shown). MleP, like CitP, preferred (S)-citramalate over (R)-citramalate indicating that the preference for a CH_2COO^- group at R_S prevails over the preference for a CH_3 at this position.

The exchange rates of the preferred enantiomers were compared to the rates of the two none chiral substrates citrate and glycolate. CitP catalyzed exchange with (S)-malate at a higher rate than with citrate, which is the physiological substrate of CitP. As demonstrated before, citrate is not transported by MleP and glycolate is a much better substrate for MleP than for CitP (6). For both transporters the rates with the preferred enantiomers of lactate were higher than with glycolate, indicating the relevance of the methyl group at the C2 atom.

Counterflow of (S)- and (R)-malate catalyzed by MleP. Heterologous exchange does not discriminate between compounds for which the transporter has no affinity and compounds that

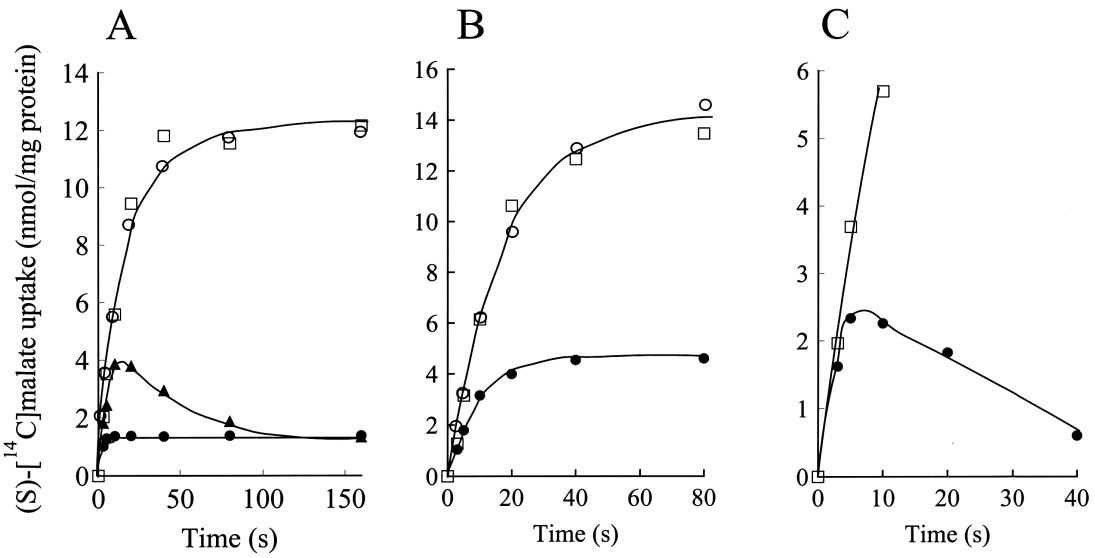


Figure 4. **Inhibition of counterflow catalyzed by MleP.** RSO membrane vesicles of *L. lactis* MG1363 expressing MleP were preloaded with 5 mM (S)-malate and diluted 100-fold into buffer containing 9.8 μM (S)-[^{14}C]malate without further additions (open squres) or with of 400 μM (S)-malate (A, closed circles), 400 μM (S)-tartrate (A, triangles), 400 mM (R)-malate (A, open cicles), 200 μM (S)-citramalate (B, closed circles), 200 μM (R)-citramalate (B,open circles) and 400 μM (S)-lactate (C, closed circles).

bind to the transporter but are not translocated. This discrimination can be made by a counterflow assay in which uptake of external labeled substrate is driven by exchange with internal unlabeled substrate. The potency of an externally added compound to inhibit the uptake of the label is a measure of the affinity of the transporter for the compound.

Right-side-out membrane vesicles containing MleP were preloaded with 5 mM unlabeled (S)-malate and, subsequently, diluted 100-fold into buffer containing 50 μM labeled (S)-malate. The label rapidly entered the vesicles, and a steady state was reached in a min (Figure 4A, squares). No subsequent release of label was observed during the time of the experiment, consistent with the low rate of efflux in the exchange experiments observed above. When the dilution buffer contained an additional 400 μM unlabeled (S)-malate, the initial rate of influx of the label was decreased due to competition between labeled and unlabeled (S)-malate. The final level of uptake was considerably lower because of the higher total external concentration of transportable substrates (Figure 4A, closed circles). Addition of 400 μM (R)-malate rather than (S)-malate had no significant effect on the uptake of the label (Figure 4A, open circles). The lack of inhibition of the initial rate of uptake indicates that MleP has a low, if any, affinity for (R)-malate. Clearly, the lack of activity with (R)-malate in the heterologous exchange assay correlates with a lack of affinity of MleP for the R-enantiomer.

In some cases an overshoot in internalized label was observed, for instance with 400 μM (S)-tartrate (Figure 4A, triangles). Initially, the rate of exchange with labeled (S)-malate is much faster than with unlabeled (S)-tartrate, but eventually, internalized label will be exchanged for (S)-tartrate until the same final level of uptake is reached as observed with 400 μM (S)-malate. The experiment demonstrates that (S)-tartrate is transported by MleP, but with a lower affinity than observed for (S)-malate.

Stereoselectivity in binding by CitP and MleP. The relative affinities of CitP and MleP for the S- and R-enantiomers of the substrates were determined from the inhibition of (S)-malate counterflow. An inhibitor concentration was selected for the different substrates that allowed estimation of the inhibition from the initial rates of label influx (Table 1).

For both CitP and MleP, the S-enantiomers of the compounds with a hydrophilic R group (i.e., malate, 2-hydroxyglutarate, and tartrate) caused a stronger inhibition than was found for the R-enantiomers (Table 1). The higher exchange rates observed above with the S- relative to

Table 1. **Inhibition of (S)-malate counterflow catalyzed by CitP and MleP[a].**

	CitP			MleP		
		Inhibition (%)			Inhibition (%)	
	[I] (mM)	S-isomer	R-isomer	[I] (mM)	S-isomer	R-isomer
malate	0.2	67	0	0.2	37	0
tartarate	4.5	27	0	0.8	39	0
2-hydroxyglutarate	5.0	27	0	10.0	33	0
lactate	6.0	13	–[b]	5.0	–[b]	25
2-hydroxyisovalerate	6.0	0	35	5.0	23	24
mandelate	5.0	0	30	2.5	43	47
citramalate	0.2	93	0	0.2	42	0
citrate	0.2	70		5.0	33	
2-hydroxyisobutyrate	1.0	33		1.25	30	
glycolate	15.0	20		5.0		16

[a] (S)-malate counterflow was done as descibed in the legend to Figure 4 with the indicated concentration of the inhibitor [I] in the dilution buffer. Inhibition is given as the decrease of the initial rate in the absence of the inhibitor. [b] initial rates could not be determined.

the R-enantiomers correlated with a higher affinity of the transporters for the S-enantiomers. (S)-Citramalate, which combines both a hydrophilic substituent at R_S and a hydrophobic substituent at R_R, was found to be a potent inhibitor of both transporters, while the R-enantiomer did not result in any inhibition at the same concentration (Figure 4B). Citrate, which has the same hydrophilic R-group at both R_S and R_R was an equally potent inhibitor of CitP. Surprisingly, citrate also inhibited counterflow catalyzed by MleP that does not transport citrate, indicating that citrate binds to MleP but is not translocated.

For MleP, the inhibition of counterflow by (S)-lactate and for CitP, the inhibition by (R)-lactate could not be accessed because of a combination of high exchange rate and relatively low affinity. For instance, at 0.4 mM (S)-lactate, the final level of uptake of label was reached very rapidly, not allowing estimation of the initial rate (Figure 4C). Relatively high concentrations of the opposite enantiomers, (R)-lactate in case of MleP and (S)-lactate in case of CitP, were required to obtain significant inhibition, suggesting low affinities.

The R-enantiomers of the compounds with the larger hydrophobic substituents (i.e., 2-hydroxyisovalerate, and mandelate) were the stronger inhibitors of counterflow catalyzed by CitP. For MleP, the inhibition by 2-hydroxyisovalerate and mandelate was the same for the two enantiomers. Since exchange activity catalyzed by MleP could not be detected with (R)-mandelate and (R)-2-hydroxyisovalerate at concentrations of 5 mM, these substrates bind to MleP but are not translocated as was concluded for citrate. The stereoselectivity of MleP observed in the exchange reaction of these compounds appears to be related to differences in turnover rate rather than affinity.

The concentrations of the 2-hydroxycarboxylates with hydrophobic R groups that are required for significant inhibition are higher than those for the preferred S-enantiomers of the physiological substrates with hydrophilic R-groups. This is also the case for the nonchiral glycolate. Nonchiral hydroxyisobutyrate, with methyl groups at R_R and R_S seems to be the most potent inhibitor in the group of substrates with the hydrophobic R substituents.

Kinetic parameters of CitP and MleP. The heterologous exchange assay and counterflow assay described above represent the same kinetic mode of the transporters, characterized by the same set of kinetic constants (see the Discussion). The K_m obtained for external (S)-malate in homologous exchange catalyzed by CitP was 0.1 mM which was in fair agreement with the K_i value of 0.08 mM obtained from the inhibition of counterflow (Table 2 and Experimental

Table 2. **Kinetic parameters of CitP and MleP.**

	CitP			MleP		
Substrate[a]	V_{max}[b] (%)	K_m[b] (mM)	K_i[c] (mM)	V_{max}[b] (%)	K_m[b] (mM)	K_i[c] (mM)
glycolate	–	–	≥ 40[d]	–	–	≥ 25[d]
(S)-lactate	110 ± 23	26 ± 8	–	140 ± 10	4.6 ± 0.9	–
(S)-malate	100 ± 3	0.10 ± 0.02	0.08 ± 0.02	100 ± 4	0.46 ± 0.15	0.40 ± 0.06
(S)-citramalate	12 ± 2[e]	–	0.014 ± 0.002	7.5 ± 0.5[e]	–	0.25 ± 0.03
citrate	69 ± 3[e]	–	0.056 ± 0.009	0	–	9 ± 2
(R)-citramalate	–	≥ 50[f]	–	–	≥ 50[f]	–
(R)-malate	–	≥ 50[f]	–	–	≥ 50[f]	–
(R)-lactate	380 ± 87	32 ± 9	15 ± 2.4	14 ± 5	–	–
glycolate	–	–	≥ 40[d]	–	–	≥ 25[d]

[a] Starting with glycolate at the top and bottom, substrates were ordered according to increasing side chains up to citrate (see also Fig. 2). [b] Maximal rate and affinity constant for the substrate in heterologous exchange with (S)-malate as described in the legend to Figure 1. Maximal rates were relative to the rate for homologous (S)-malate exchange that was set at 100. [c] Inhibition constant for the substrate inferred from the inhibition of (S)-malate counterflow as described in the legend to Figure 3. The K_i values were calculated using a K_m value for (S)-malate of 90 μM (CitP) or 0.43mM (MleP) . [d] Estimated from the data in Table 1. [e] Maximal rates were the exchange rates at a substrate concentration that was 20 times the K_i value obtained from the inhibition of counterflow. [f] Lower limit of the affinity constants. No exchange activity could be measured.

procedures). A similar result was obtained with MleP, but the K_m and K_i values of 0.46 and 0.4 mM, respectively, showed that the affinity of MleP for (S)-malate was approximately 5 times lower than of CitP. Estimation of the kinetic parameters for (R)-malate was hampered by the presence of a transportable contamination, most likely (S)-malate. No significant inhibition of counterflow was observed at concentrations up to about 1 mM. Moreover, in the heterologous exchange assay, using a concentration of 5 mM (R)-malate, the rate and extend of exchange correlated with the presence of about 100 μM (S)-malate in the external medium (Figure 1, triangles), suggesting no inhibition by (R)-malate at this concentration. The affinity of CitP and MleP for (R)-malate can be estimated to be at least 2 orders of magnitude lower than for (S)-malate.

The low exchange rates with (S)-citramalate did not allow a reliable estimation of the affinity of the transporters from a titration of the external (S)-citramalate concentration. Therefore, the affinity constant was estimated from the inhibition of counterflow at a range of concentrations. (S)-Citramalate is the substrate for which both CitP and MleP have the highest affinity with K_i values of 14 and 250 μM, respectively. The maximal exchange rates with (S)-citramalate were estimated from the exchange rate at an external concentration that was 20 times higher than the inhibition constants. The V_{max} values were roughly 10 times lower than those for (S)-malate. Analysis of the kinetics of (R)-citramalate was not possible because of the presence of contaminating (S)-citramalate. Similar experiments as described above for (R)-malate suggested that the affinity of both transporters for (R)-citramalate was very low.

The affinity of CitP and MleP for citrate was estimated from the inhibition of (S)-malate counterflow. The affinity of CitP (K_i = 56 μM) was in the same range as observed for (S)-malate and (S)-citramalate. The maximal rate of heterologous exchange was determined at a citrate concentration of 5 mM and found to be somewhat slower than that with (S)-malate. Titration of the inhibition of counterflow catalyzed by MleP revealed an inhibition constant K_i of 9 mM for citrate. No exchange activity could be measured with citrate concentrations twice the K_i value, showing that citrate binds to MleP but is not translocated.

The kinetic constants for both stereoisomers of lactate were estimated from heterologous exchange of (S)-malate with external lactate concentrations ranging from 0.5 to 20 mM (Figure

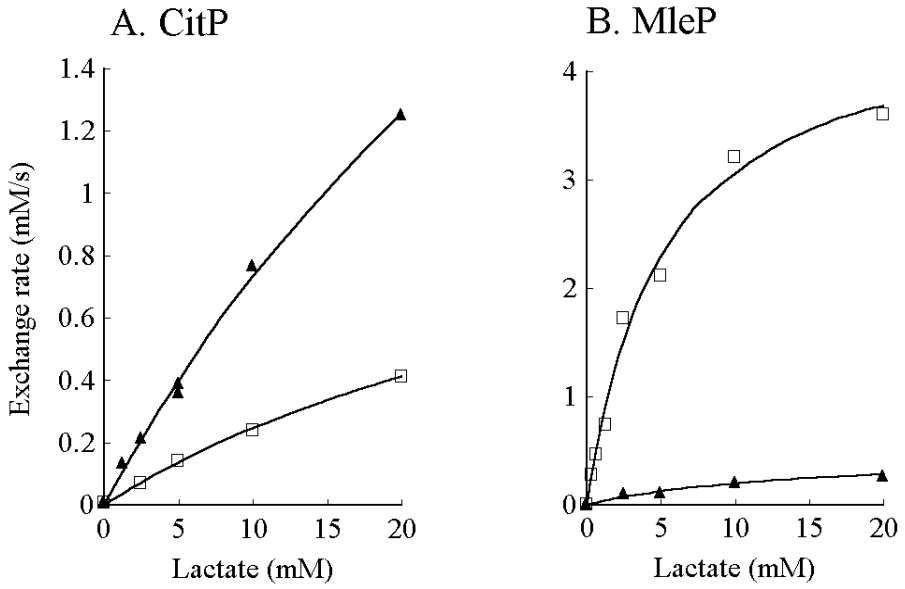


Figure 5. **Kinetic differences between MleP and CitP for (S)- and (R)-lactate exchange**. RSO membrane vesicles of *L. lactis* MG1363 expressing CitP (A) and MleP (B) were preloaded with 5 mM (S)-[^{14}C]malate. The membranes were diluted 100-fold into buffer containing concen-trations of (R)-lactate (triangles) and (S)-lactate (open squares) ranging from 1 to 20 mM. Heterologous exchange rates were plotted against the lactate concentrations. For the kinetic parameters derived from the plots, see Table 2.

5). The affinity constants of CitP for (S)- and (R)-lactate were very similar, 26 and 32 mM, respectively, while the maximal rate with (R)-lactate was almost 4 times higher than with (S)-lactate (Table 2). The affinities of MleP for lactate were higher than those of CitP. (S)-Lactate resulted in a 3-fold higher affinity than (R)-lactate (4.6 vs 13.8 mM). The most prominent difference was the 10-fold higher maximal rate for (S)-lactate when compared to that of (R)-lactate.

Glycolate at a concentration of 5 mM exhibited significant rates of exchange with (S)-malate for both CitP and MleP. The level of inhibition of counterflow at this concentration was low, suggesting much higher affinity constants and high maximal rates. Precise measurement of K_m and V_{max} was not possible because higher concentrations started to inhibit exchange nonspecifically and due to the increased ionic strength of the buffer. The affinity constant for glycolate of both CitP and MleP can safely be estimated to be higher than 40 mM for CitP and 25 mM for MleP.

Role of the 2-hydroxycarboxylate motif in binding. CitP and MleP translocate substrates with the 2-hydroxycarboxylate motif. Exceptions to the rule are oxaloacetate that is translocated efficiently by CitP and the 3-hydroxycarboxylates 3-hydroxybutyrate and 2,2-dimethyl-3-hydroxypropionate that are translocated by both transporters, albeit at a slow rate (6). Inhibition of counterflow showed that a similar degree of inhibition required a concentration of the 2-oxocarboxylate oxaloacetate 50 times higher than that of the corresponding 2-hydroxycarboxylate (S)-malate (not shown). A similar difference was obtained between 2-hydroxyisobutyrate and 2,2-dimethyl-3-hydroxypropionate. Replacing the hydroxyl in citrate, malate and citramalate with hydrogen (yielding tricarballate, succinate and methylsuccinate, respectively) resulted in complete loss of affinity as inferred from the lack of inhibition of these compounds at 20 mM concentrations in the counterflow assay. At the least, the hydroxyl group is essential for high-affinity binding.

Methylation of the carboxylate in (S)-lactate to methyl-(S)-lactate and reduction to glyceraldehyde and 1,2-propaandiol resulted in complete loss of affinity in the counterflow assay (not shown), indicating that the carboxylate group of the motif is essential for binding.

DISCUSSION

CitP and MleP are homologous transporters capable of transporting a range of 2-hydroxycarboxylates of different sizes and charges. Under physiological conditions, the proteins exchange a divalent precursor (citrate or malate) present in the medium for the monovalent metabolic product of the precursor (lactate) that is present in the cytoplasm. Consequently, they generate a membrane potential, supplying the cell with metabolic energy, and take part in the removal of the end product from the cell. CitP and MleP are believed to be "normal" secondary transporters that have been optimized to catalyze exchange (1). The transporters have been shown to be H^+/substrate symporters (5, 7). Symport involves binding, translocation, and dissociation of the substrates in one direction and reorientation of the empty binding sites in the reverse direction. In the exchange mode, the second step is replaced by the same sequence as in the first step but in the opposite direction. In exchangers like CitP and MleP, reorientation of the empty binding sites is slow relative to reorientation of the substrate-bound sites, resulting in much higher rates for exchange than for unidirectional modes of transport, like efflux (for instance, see Figure 1). The heterologous exchange assay and counterflow assay used in this study both measure exchange activity of the transporters, but in different experimental setups. In the former, turnover is assessed by following the release of radiolabeled internal (S)-malate from the membranes, while in the latter, uptake of external radio labeled (S)-malate is assessed. Turnover of the transporters was assessed at a constant internal (S)-malate concentration and a variable external substrate/inhibitor composition. Measurement of the exchange rates at different external substrates concentrations revealed both the affinity constant K_m for the external substrate and the maximal heterologous exchange rate V_{max}. Measurement of the extent of inhibition of counterflow at different external substrate concentrations revealed the inhibition constant K_i of the substrate. When corrected for the external (S)-malate concentrations (see Experimental Procedures), the affinity constants from the two measurements represent the same parameter as is nicely demonstrated for (S)-malate, in which case experimental conditions allowed the measurement of the affinity constant using both assays (Table 2).

The maximal initial rate of exchange is determined by the reorientation and dissociation of the enzyme-substrate complexes in the two directions. In the heterologous exchange and counterflow assays used here, translocation of (S)-malate from "in" to "out" is common to all measurements, while the different substrates in the dilution buffer result in different complexes that translocate from "out" to "in". Since the maximal rate of (R)-lactate-(S)-malate exchange catalyzed by CitP was 4 times faster than observed for homologous (S)-malate exchange, it can be concluded that the common translocation of (S)-malate from "in" to "out" is not rate controlling (Table 2). The translocation and/or dissociation step of (S)-malate, citrate and (S)-citramalate into the vesicle is rate determining at saturating concentrations of the substrates. It was shown before that entrance of citrate into the cell catalyzed by CitP is the rate-controlling step in the citrate metabolic pathway in *Lc. mesenteroides* (8).

Citrate-lactate and malate-lactate exchange are the physiological modes of transport of CitP and MleP, respectively. The main metabolic activity of lactic acid bacteria is the conversion of carbohydrates in lactate that accumulates in the medium. Eventually, lactate in the medium will inhibit the citrate- and malate-degrading pathways since the transporter has affinity for both citrate or malate and lactate. This study shows that CitP and MleP have affinities for the di/tricarboxylates malate and citrate that are at least one order of magnitude higher than the affinity for the monocarboxylate lactate at the external face of the membrane. The difference in affinity largely prevents the inhibition of the citrate and malate pathways by the accumulating lactate in the growth medium (see also ref 20).

The affinity and translocation properties determined in this study result in a model for the substrate binding site of the CitP and MleP proteins. With few exceptions, both transporters were known to transport substrates containing the 2-hydroxycarboxylate motif (6). The present

study shows that the inability to transport substrates in which either the hydroxyl or the carboxylate of the motif were modified is due to the loss of binding affinity. It seems reasonable to assume that CitP and MleP recognize their substrates through an interaction with both functional groups. Under physiological conditions the carboxylate group is negatively charged, and since removal of the charge resulted in complete loss of affinity, the interaction is likely to be electrostatic. The hydroxyl group could, to some extent, be replaced by an oxo group (for instance oxaloacetate), be it with significant loss of affinity. Possibly, the hydroxyl is hydrogen bonded to the protein with the oxygen atom acting as the H-acceptor. Alternatively, the 2-oxo substrates are bound in their hydrated form in which the 2-hydroxy group is restored. Flexibility in the substrate molecule and/or the protein may account for the low activity observed with 3-hydroxycarboxylates like 3-hydroxybutyrate (6).

The interactions of the transporters with the 2-hydroxycarboxylate motif fix the substrates in the binding pocket allowing a detailed analysis of the effect of the two R groups. In the two enantomeric forms of monosubstituted 2-hydroxycarboxylates, i.e., HO-CHR-COO^-, the same R group has a different spatial orientation relative to the hydroxyl and carboxyl groups of the motif when bound to the protein (see Figure 2). For hydrophilic R groups, CitP and MleP interact efficiently with the S-enantiomers, while interaction with the R-enantiomers could not be detected. All the substrates in the micomolar affinity range [(S)-malate, (S)-citramalate and citrate] have a CH_2COO^- group at the R_S position, suggesting that this group or, more particularly, the negatively charged carboxylate is essential for high-affinity binding via an electrostatic interaction with the protein. The high affinity binding of di- and tricarboxylates as compared to monocarboxylates is of physiological relevance as discussed above. The lower affinities for the dicarboxylates (S)-tartrate and (S)-2-hydroxyglutarate are due to additional features of the R groups (Table 1 and Figure 2). Apparently, the additional hydroxyl in (S)-tartrate [as compared to (S)-malate] results in a negative interaction with the proteins, while the extended length of the R group in (S)-2-hydroxyglutarate wrongly positions the carboxylate in the binding site. The inability of especially CitP to interact with substrates with a second carboxylate at the R_R position is not due to a spatial restriction in the binding site since citrate, with CH_2COO^- groups at both R_S and R_R, is a good substrate. Citrate however is known to be transported in its divalent negative form (5), leaving one of the three carboxylates uncharged. Since (S)-malate is also transported in its divalent form, the protonated carboxylate in citrate is likely to be at the R_R position. This suggest that specifically a negative charge is not tolerated at the R_R position in CitP, for instance, because of the presence of a hydrophobic surface in the binding pocket.

The affinity of both CitP and MleP for the substrate increases when a methyl group is present at the R_S and R_R positions as evidenced by the improved affinities of (S)- and (R)-lactate relative to those of glycolate (Table 2). The effects of the methyl groups at R_S and R_R are additive since 2-hydroxyisobutyrate with methyl groups at both positions showed higher affinity than (R)- en (S)-lactate. Addition of the methyl to (S)-malate at the R_R position, i.e., (S)-citramalate, also resulted in significant higher affinity. Apparently, the methyl groups give a better fit of the substrates in the binding site resulting in an improved interaction with the protein.

The stereoisomers of the monosubstituted substrates with hydrophobic R groups behaved in a manner different from those with the hydrophilic R groups. In addition, these substrates revealed differences between the binding pockets of MleP and CitP. In marked contrast to the substrates with the hydrophilic R groups, MleP bound the R- and S-enantiomers of lactate, 2-hydroxyisovalerate and mandelate with similar affinity. The higher affinity of mandalate relative to those of the two other substrates indicates the presence of hydrophobic surfaces in the binding pocket and no steric restrictions to accommodate a benzyl group at both the R_S and R_R positions. A marked difference between the R- and S-enantiomers was evident in the translocation step. (S)-Lactate is translocated 10 times faster than (R)-lactate (Figure 5 and Table 2) while translocation of mandalate and 2-hydroxyisovalerate could only be detected in

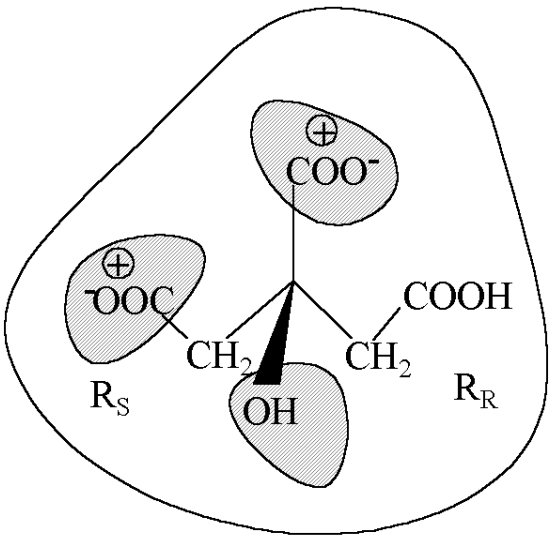


Figure 6. **Model of the substrate binding site of CitP and MleP.** Relevant interactions between substrate molecule and the protein are indicated by gray surfaces. The substrate depicted in the pocket is citrate. Steric restrictions during translocation at R_R prevent turnover of citrate in MleP.

the S-enantiomeric form (Figure 3). Whereas the outward facing binding site of MleP does not discriminate between the stereoisomers, the transition complex is clearly more tolerant of R groups at the R_S position than at the R_R position. CitP binds (S)- and (R)-lactate with similar affinities, but no affinity was detected for the (S)-enantiomers of 2-hydroxyisovalerate and mandelate, suggesting that CitP is spatially restricted at the R_S position. In the transition complex, CitP is very tolerant to hydrophobic R groups at R_R as evidenced by the highest of all maximal rates obtained with (R)-lactate and rates similar to those obtained with malate and citrate for (R)-mandalate and (R)-2-hydroxyisovalerate. In conclusion, MleP has difficulties accepting hydrophobic R groups at the R_R position during translocation and CitP does not bind substrates with large hydrophobic R groups at the R_S site. The conclusion explains why citrate is a substrate of CitP and not of MleP. The CH_2COOH group of citrate at the R_R position is bound and translocated by CitP, while MleP can accept the group in the binding site, but does not allow translocation (Table 2).

In summary, the substrate binding pockets of CitP and MleP are found to be very much alike, especially where the properties are concerned that give the transporters their physiological function as precursor-product exchangers. The binding pocket contains sites that specifically interact with the carboxylate and the hydroxyl of the 2-hydroxycarboxylate motif that is common to precursors and product (Figure 6). A separate site in the pocket interacts with the second carboxylate of the S-enantiomers of di- and tricarboxylates, resulting in a high affinity for the precursors and a strong stereoselectivity for dicarboxylates. Both interactions with the carboxylate groups may be mediated by positively charged amino acid residues on the protein. At the R_R, a hydrophobic surface rejects the carboxylates of the R-enantiomers of dicarboxylates and contributes to the affinity for substrates with large hydrophobic substituents. Optimal interaction with the substrate at the R_S and R_R positions in the pocket seems to require at least methyl groups. The difference in stereoselectivity between CitP and MleP for monocarboxylates was found to be due to differences in both affinity and translocation properties. CitP is quite tolerant of larger groups at the R_R position, and less tolerant of those at R_S, while MleP can accommodate larger groups at both R_S and R_R; however, the presence of a group at R_R prevents translocation.

Substrate binding by MleP and CitP shows some interesting similarities to substrate binding by the Na^+/dicarboxylate cotransporter (NaDC-1) from renal brush borders (21, 22). Simmilar to CitP and MleP, this transporter has a broad substrate specificity and its preferred substrates are divalent anions. Moreover it was found that one carboxylate on the substrate is essential for binding and the other increases the affinity. Despite the absence of sequence homology between NaDC-1 and the MleP and CitP proteins certain basic principles for binding and translocating carboxylic acids may apply.

Future studies on MleP and CitP will include a detailed investigation of mutant transporters to identify the amino acid residues involved in the different interactions with the substrate as defined in this study.

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REFERENCES

1. Lolkema, J. S., Poolman, B., and Konings, W. N. (1996) in *Handbook of Biological Physics* (Konings, W. N., Kaback, H. R., and Lolkema, J. S. eds) pp. 229-260, Elsevier, Amsterdam
2. Anantharam, V., Allison, M. J., and Maloney, P. C. (1989) *J. Biol. Chem. 264*, 7244-7250
3. Fu, D. and Maloney, P. C. (1997) *J. Biol. Chem. 272*, 2129-2135
4. Fu, D. and Maloney, P. C. (1998) *J. Biol. Chem. 273*, 17962-17967
5. Marty Teysset, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1995) *J. Biol. Chem. 270*, 25370-25376
6. Bandell, M., Ansanay, V., Rachidi, N., Dequin, S., and Lolkema, J. S. (1997) *J. Biol. Chem. 272*, 18140-18146
7. Poolman, B., Molenaar, D., Smid, E. J., Ubbink, T., Abee, T., Renault, P. P., and Konings, W. N. (1991) *J. Bacteriol. 173*, 6030-6037
8. Marty Teysset, C., Posthuma, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1996) *J. Bacteriol. 178*, 2178-2185
9. David, S., van der Rest, M. E., Driessen, A. J. M., Simons, G., and De Vos, W. M. (1990) *J. Bacteriol. 172*, 5789-5794
10. Vaughan, E. E., David, S., Harrington, A., Daly, C., Fitzgerald, G. F., and De Vos, W. M. (1995) *Appl. Environ. Microbiol. 61*, 3172-3176
11. Bandell, M., Lhotte, M. E., Marty Teysset, C., Veyrat, A., Prevost, H., Dartois, V., Divies, C., Konings, W. N., and Lolkema, J. S. (1998) *Appl. Environ. Microbiol. 64*, 1594-1600
12. van der Rest, M. E., Siewe, R. M., Abee, T., Schwarz, E., Oesterhelt, D., and Konings, W. N. (1992) *J. Biol. Chem. 267*, 8971-8976
13. Pos, K. M. and Dimroth, P. (1996) *Biochemistry 35*, 1018-1026
14. van Geest, M. and Lolkema, J. S. (1996) *J. Biol. Chem. 271*, 25582-25589
15. Pos, K. M., Bott, M., and Dimroth, P. (1994) *FEBS Lett. 347*, 37-41
16. van Geest, M., Nilsson, I, von Heijne, G., and Lolkema, J. S. (1999) *J. Biol. Chem. 274*, 2816-2823
17. Lopez de Felipe, F., Magni, C., de Mendoza, D., and Lopez, P. (1995) *Mol. Gen. Genet. 246*, 590-599
18. Otto, R., Lageveen, R. G., Veldkamp, H., and Konings, W. N. (1982) *J. Bacteriol. 149*, 733-738
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275
20. Magni, C., de Mendoza, D., Konings, W. N., and Lolkema, J. S. (1999) *J. Bacteriol. 181*, 1451-1457
21. Wright, S.H., Kippen, I., Klinenberg, J.R., and Wright, E.M. (1980) *J. Membr. Biol. 57*, 73-82
22. Pajor, A.M. (1999) *Annu. Rev. Physiol. 61*, 663-682

Chapter 5

The Conserved C-Terminus of the Citrate (CitP) and Malate (MleP) Transporters of Lactic Acid Bacteria is Involved in Substrate Recognition

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SUMMARY

The membrane potential-generating transporters CitP of *Leuconostoc mesenteroides* and MleP of *Lactococcus lactis* are homologous proteins with 48% identical residues that catalyze citrate-lactate and malate-lactate exchange, respectively. The two transporters are highly specific for substrates containing a 2-hydroxycarboxylate motif ($HO\text{-}CR_2\text{-}COO^-$)in which substitutions of the R-groups are tolerated well. Differences in substrate specificity between MleP and CitP are based on subtle changes in the interaction of the protein with the R-groups affecting both binding and translocation properties. The conserved, 46-residues long C-terminal region of the transporters containing the C-terminal putative transmembrane segment XI was investigated for its role in substrate recognition by constructing chimeric transporters. Replacement of the C-terminal region of MleP for that of CitP and vice versa did not alter the exchange kinetics with the substrates malate and citrate, indicating that the main interactions between the proteins and di- and tricarboxylate substrates were not altered. In contrast, the interaction of the proteins with the monocarboxylate substrates mandelate and 2-hydroxyisovalerate changed in a complementary manner. The affinity of CitP for the S-enantiomers of these substrates was at least 1 order of magnitude lower than observed for MleP. Introduction of the C-terminal residues of MleP in CitP resulted in a higher affinity and vice versa. Interchanging the C-termini had a more complicated effect on the R-enantiomers, affecting different kinetic parameters with different substrates, indicating multiple interactions of the R-groups at this side of the binding pocket. It is suggested that the binding pocket is located between transmembrane segment XI and the other transmembrane segments of the transporters.

INTRODUCTION

The homologous citrate and malate transporters CitP and MleP found in the lactic acid bacteria *Leuconostoc mesenteroides* (1, 2) and *Lactococcus lactis* (3, 4) function in secondary metabolic energy-generating pathways. The transporters catalyze the uptake of a substrate into the cell coupled to the exit of a metabolic end product (precursor-product exchange; for a review see 5). CitP exchanges divalent citrate and MleP divalent malate for monovalent lactate, an end product of both citrate and malate degradation in lactic acid bacteria (4, 6). Net charge movement over the membrane during exchange results in a membrane potential of physiological polarity. This, in combination with the generation of a pH gradient across the membrane due to the consumption of a cytoplasmic proton during the breakdown of citrate and malate, results in a proton motive force, and thus, the pathways generate metabolic energy for the cell. More recently, the pathways have been implicated in the recovery from acidic stress and resistance against lactate toxicity (7).

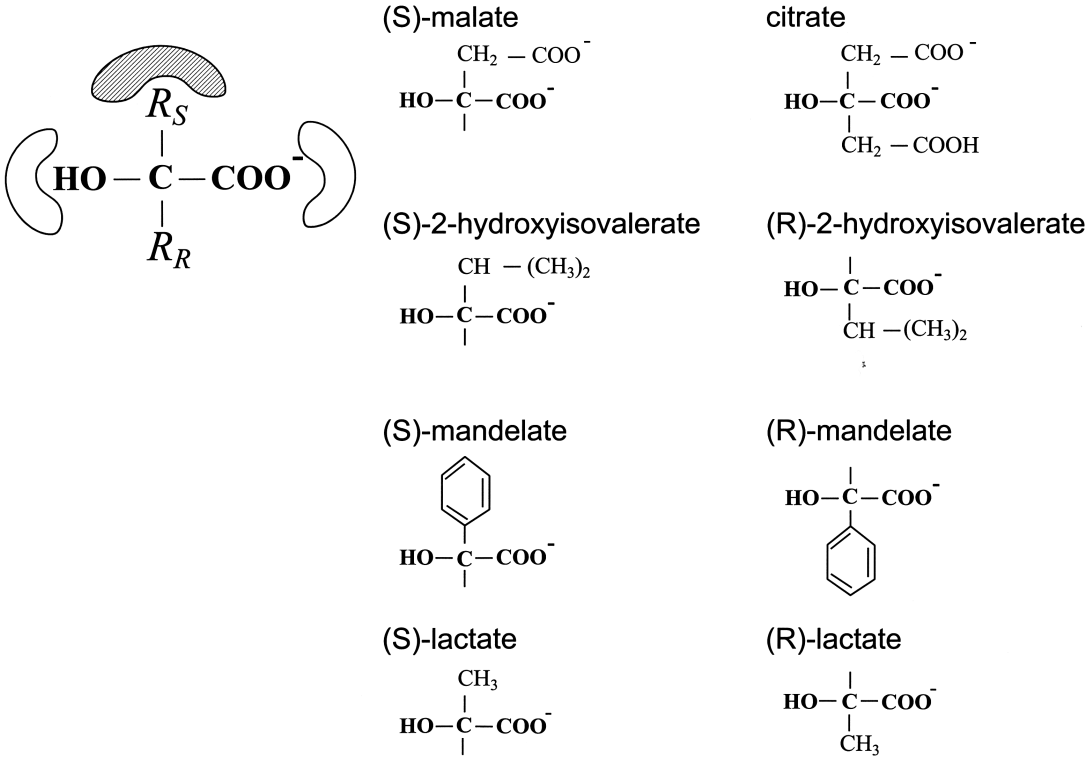

Figure 1. **Model of the substrate binding site and structure of substrates of CitP and MleP.** Sites on the proteins interacting with the essential OH and COO^- groups of the 2-hydroxycarboxylate motif are depicted as white surfaces. The interaction site responsible for high affinity binding of di- and tricarboxylates is depicted as a hatched surface. The two R-groups are indicated as R_R and R_S according to the position of the R-group in the R- and S-enantiomers of monosubstituted 2-hydroxycarboxylates (HO-CHR-COO^-). At the right are chemical structures of the substrates used in this study: malate, citrate, lactate, mandelate and 2-hydroxyisovalerate. The 2-hydroxycarboxylate motif is bold.

The substrates of CitP and MleP are structurally related (see Figure 1). The ability to exchange citrate or malate for lactate is associated with a high specificity for the 2-hydroxycarboxylate motif present in the substrates, i.e. HO-CR_2-COO^-, together with a low specificity for the two R-groups (3). Next to the physiological substrates, CitP and MleP translocate a wide range of 2-hydroxycarboxylates with R groups ranging from hydrogen atoms in glycolate to a phenyl group in mandelate (3). A detailed kinetic analysis of the two transporters with different substrates showed that interactions with the OH and COO^- groups of the motif are essential for binding. The interactions with the two R groups (defined as R_R and R_S; see Figure 1) are not essential but modulate affinity and translocation efficiency (8). The tolerance towards the R-groups gives the transporters their membrane potential-generating capacity. Two types of interactions take place between the R groups and the binding sites of CitP and MleP, depending on the nature of the R-group (8). A strong, possibly electrostatic interaction results in high affinity for 2-hydroxycarboxylates bearing an additional carboxylate group at R_S (see Figure 1). For example, the S-enantiomer of malate is a high-affinity substrate. Noncharged R groups have a less strong, most likely hydrophobic interaction with the binding site, resulting in low affinity for monocarboxylates, like lactate.

The difference in substrate recognition by CitP and MleP is caused by subtle differences in the interactions with the R groups of the substrates. Most prominent are (i) the difference in affinity between the mono- and dicarboxylates which is 1 order of magnitude higher for CitP than for MleP and (ii) CitP is more promiscuous in the translocation step toward larger R groups than MleP. The latter feature is responsible for the physiological function of the two transporters as citrate and malate transporters; CitP translocates both citrate and malate, while

MleP translocates malate but cannot translocate the larger citrate molecule.

CitP and MleP are 48% identical in their amino acid sequences and belong to the 2-hydroxycarboxylate transporter (2-HCT) family (3). The membrane topology model of these transporters consists of 11 transmembrane segments (TMS), based upon studies of the citrate transporter CitS of *Klebsiella pneumoniae,* which also belongs to the 2-HCT family (9-11). The C-terminal part of the amino acid sequences of the members of the 2-HCT family, including TMS XI and part of the cytoplasmic loop between TMS X and XI, is particularly well conserved. Since the main interactions with the substrates are very similar for CitP and MleP (8), this suggests that structural elements involved in substrate binding may be located in this part of the proteins (3). In the present study we have constructed chimeric CitP-MleP transporters in which the conserved C-terminal parts of the proteins were interchanged. The chimeras were analyzed for their catalytic properties with 2-hydroxycarboxylate substrates bearing different R-groups to locate the interactions between the proteins and the functional groups of the substrates in the C- or N-terminal part of the chimeras. The results suggest that the C-terminal 46 residues of MleP and CitP contain structural elements that interact with the R groups of the substrates.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. *L. lactis* strain NZ9000 is a MG1363 derivative (*pepN*::*nisRnisK*; 12) that does not transport citrate and malate. The *nisR* and *nisK* genes were inserted into the chromosome to allow induced expression of plasmid-encoded citrate and malate transporters under control of the tightly regulated nisA promoter (12). Cells were grown at 30 °C in closed serum bottles and without shaking. The growth medium contained M17 broth (Difco) supplemented with 0.5% (w/v) glucose and 5 μg/mL chloramphenicol. Cells were induced by adding supernatant of the nisin producing *L. lactis* strain NZ9700 (13), containing approximately 10 ng of nisin A/mL (14), to the cultures at an OD_{660} of 0.6. Typically, a 1000-fold dilution of the supernatant was added after which growth was continued for 1 h followed by harvesting of the cells by centrifugation. In some cases a lower induction was used to allow kinetic analysis (see the Results). Then, cells expressing CitP, MleP or MC397 were grown in the presence of a 30,000, 50,000 and 30,000-fold dilution of the nisin-containing supernatant, respectively, for 0.5 h.

Construction of Expression Vectors and Chimeras. General procedures for cloning and DNA manipulations were performed essentially as described by Sambrook et al. (15). Ligation mixtures were transformed to *L. lactis* NZ9000 using a electroporation protocol described by Holo and Nes (16). Expression vector pGltThis (17) encodes the glutamate transporter of *Bacillus stearothermophilus,* with an N-terminal extension of 6 histidine residues (His tag) followed by a enterokinase proteolytic cleavage site. The coding region was amplified by the polymerase chain reaction technique (PCR) using a forward primer (5'-ATTT**ACATGT**CGCATCACCATCA-CCATCACCATCACCATCACG-3'), containing a *Afl*III restriction site (bold) and 10 histidine codons (underlined), and a reverse primer downstream of the stop codon, containing a *Xba*I site (17). The *Afl*III-*Xba*I-digested PCR product was ligated into the *Nco*I-*Xba*I sites of the nisin inducible expression vector pNZ8048 (Cm^R, pSH71 replicon, inducible *nisA* promoter; O. Kuipers, unpublished), yielding pNZ*gltT*. Successful ligation destroys the *Nco*I site at this position. Subsequently, the *gltT* gene was removed, using the *Nco*I site just downstream of the enterokinase site (15) and the *Xba*I site downstream of the stop codon, and replaced by the *citP* and *mleP* containing *Nco*I-*Xba*I fragments from plasmids pMB*citP* and pMB*mleP* that were described previously (3). The resulting expression vectors, pNZ*citP* and pNZ*mleP* encode the CitP and MleP transporter, respectively, extended with a 10-His tag and a enterokinase site at the N-terminus. The amino acid sequences of the N-terminal parts of CitP and MleP were MSHHHHHHHHHHDDDDKA**MVNHP**.... and MSHHHHHHHHHHDDDDKA**M-**GK**KLK**....**,** respectively, in which original residues are in bold and the enterokinase site is underlined.

Chimeric transporters were constructed in two steps by overlap extension PCR (18) using the pNZ*mleP* and pNZ*citP* vectors as templates. The PCR products encoding the chimeric proteins were cloned into the *Nco*I-*Xba*I sites of pNZ8048 in the same way as described above for the wild type genes. All constructs were verified by nucleotide sequencing.

Preparation of membrane vesicles, and exchange and counterflow assays. *L. lactis* NZ9000 cells expressing the wild-type or chimeric transporters were cooled on ice and washed twice with 50 mM potassium phosphate (pH 6). Right-side-out (RSO) membrane vesicles were prepared by the osmotic shock lysis procedure essentially as described previously (8). Membrane vesicles were washed once in 50 mM potassium phosphate (pH 6) containing 5 mM (S)-malate and concentrated by centrifugation for 15 min in an Eppendorf tabletop centrifuge operated at full speed, followed by resuspension in the same buffer. For exchange measurements, the internal pool of (S)-malate was labeled with (S)-[^{14}C]malate by incubating the concentrated membranes with 186.7 μM L-[1,4(2,3)^{14}C]malate for 1 h at room temperature in the presence of 100 μM valinomycin and 50 μM nigericin.

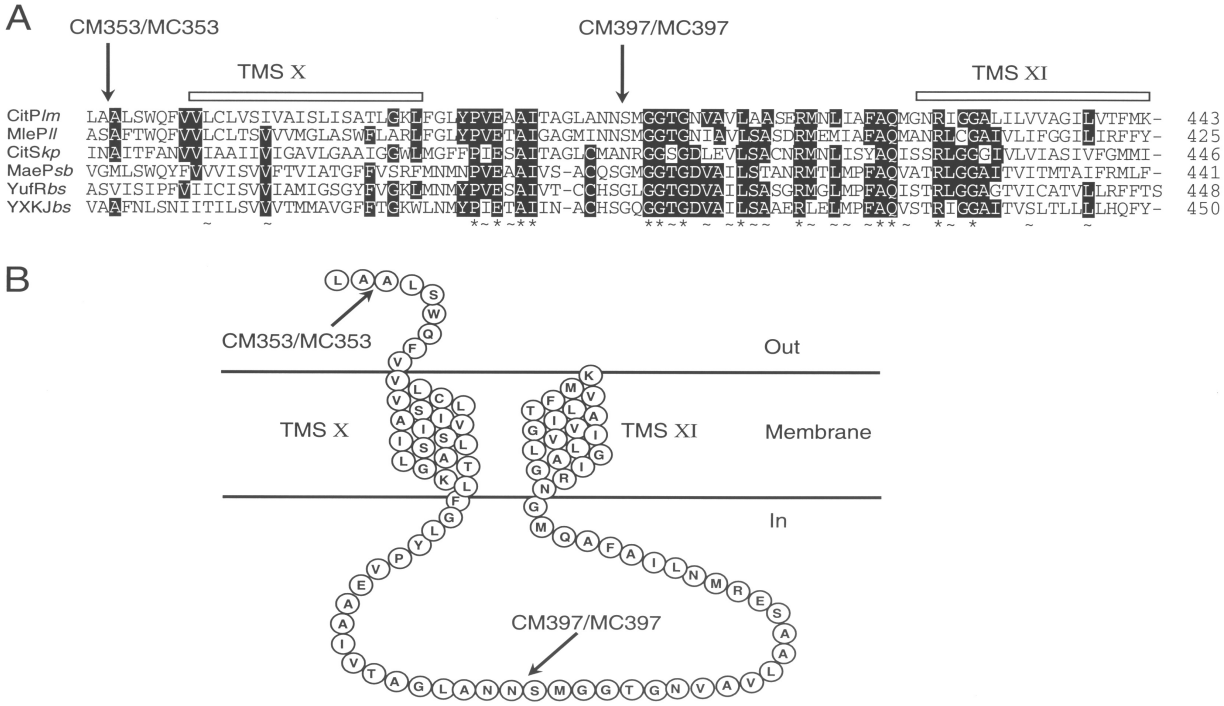

Figure 2. **Amino acid sequence alignment (A) and topology model (B) of the C-terminal part of representative members of the 2-HCT family.** (A). Transporters included in the alignment were the citrate transporter of *Lc. mesenteroides* CitP*lm* (2), the malate transporter of *L. lactis* MleP*ll* (3), the citrate transporter of *K. pneumoniae* CitS*kp* (23), the malate transporter of *Streptococcus bovis* MalP*sb* (24), and two open reading frames found on the *Bacillus subtilis* chromosome, YufR*bs* and YxkJ*bs* (25). Horizontal bars indicate putative TMSs. Arrows indicate fusion points in the chimeric proteins. Identical residues and similar residues are indicated as * and ~, respectively. Residues that are conserved in the majority of the transporters are indicated in black boxes. (B). Topology model of the C terminal part of CitP based on recent topology studies of CitS*kp* (9-11).

Aliquots (2 µL) were diluted into 200 µL of 50 mM potassium phosphate (pH 6) containing various substrates at the indicated concentrations and at 20 °C. For counterflow experiments, the (S)-malate loaded concentrated vesicles were diluted 100-fold into 200 µl buffer containing 9.8 µM L-[1,4(2,3)^{14}C]malate and different concentrations of various substrates when indicated. Final membrane protein concentrations in the assays were between 250 and 350 µg/mL. Reactions were stopped at the indicated times by addition of 4 mL ice-cold 0.1 M LiCl, followed by rapid filtration over 0.45 µm pore size cellulose nitrate filters (Schleicher & Schuell). The filters were rinsed once with buffer and transferred to scintillation vials to determine the retained radioactivity. Protein concentrations were determined as described by Lowry et al. (19).

Evaluation of the data. Initial rates of exchange were determined by fitting the data to an exponential decay as described previously (3) using nonlinear fitting procedures provided with Sigma Plot (Jandel Scientific, San Rafael, CA). Exchange rates were determined at different external substrate concentrations. The affinity constant (K_m) for the substrate was determined by fitting the data to an equation describing competitive inhibition that takes into account the concentration of (S)-[^{14}C]malate in the external buffer caused by the dilution of the (S)-malate-loaded vesicles (see ref 8). Initial rates of counterflow were estimated from internalized label measured in triplicate at 2 and 4 s. Data were used only when the uptake increased proportional with time to assure initial rate conditions. Inhibition of (S)-malate counterflow by a substrate was assessed at different concentrations of the substrate in the external buffer. The inhibition constant (K_i) for the substrate was estimated as described previously (8) by fitting the data to an equation describing competitive inhibition. When the affinity for the substrate was low, K_i values were estimated from a single concentration of 20 mM measured at least in triplicate. A lower limit for the K_i was calculated when the inhibition was less than 10%. A K_m for exchange or a K_i for counterflow was determined, depending on the transport properties of the substrate (8).

SDS-PAGE and Immunoblot Analysis. Right-side-out membrane vesicles (15 µg protein/lane) were subjected to SDS-PAGE using a 12% polyacrylamide gel matrix. Following electrophoresis, the proteins were transferred to poly(vinylidenedifluride) membranes and analyzed using monoclonal antibodies directed against a His-tag (Dianova, Hamburg, Germany). Antibodies were visualized using the Western-light chemiluminesence detection kit (Trophix, Bedford, MA)

Materials. L-[1,4(2,3)-^{14}C]malic acid (51 mCi/mmol) and [1,5-^{14}C]citric acid (115 mCi/mmol) were obtained from Amersham International (Buckinghamshire, U.K.). All other compounds were obtained from Fluca (Buchs, Switzerland) or Sigma (St. Louis, MO).

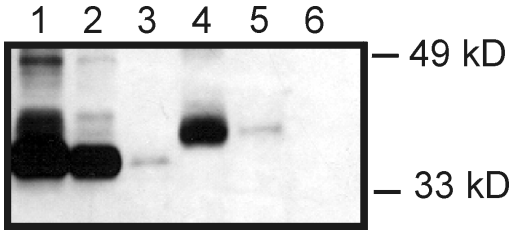

Figure 3. **Expression levels of wild-type and chimeric transporters.** Immunoblot of RSO membrane vesicles prepared from *L. lactis* NZ9000 expressing the 10-His-tagged MleP (lane 1), MC397 (lane 2), MC353 (lane 3), CitP (lane 4), CM397 (lane 5), CM353 (lane 6). Indicated on the right are the molecular mass markers.

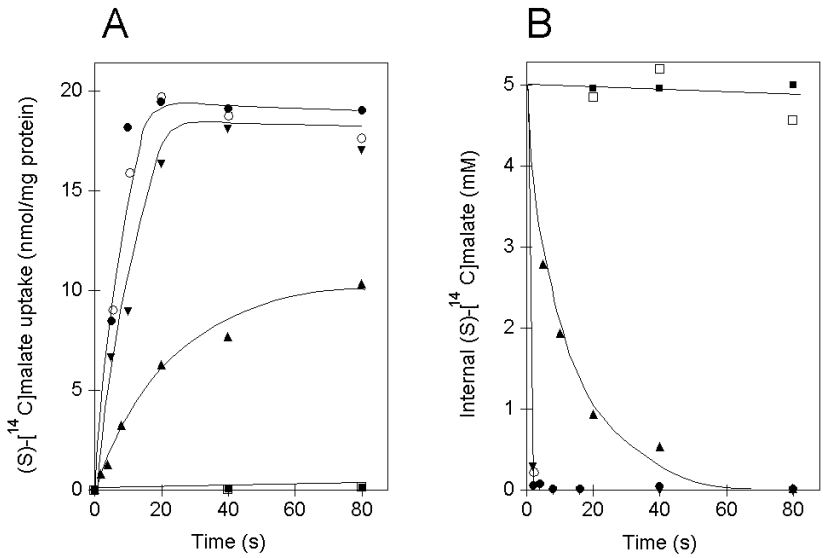

Figure 4. **Homologous (S)-malate counterflow (A) and exchange (B) by wild-type and chimeric transporters.** RSO membrane vesicles of *L. lactis* NZ9000 expressing CitP (closed circles), MleP (open circles), CM397 (triangles), MC397 (wedges), CM353 (closed squares), MC353 (open squares) were preloaded with 5 mM (S)-malate and diluted 100-fold into buffer containing 9.8 μM (S)-[^{14}C]malate (A) or preloaded with 5mM (S)-[^{14}C]malate and diluted 100-fold into buffer containing 5 mM (S)-malate (B).

RESULTS

Construction of Chimeric Transporters. Figure 2 shows a multiple-sequence alignment of the C-terminal part of the members of the 2-hydroxcarboxylate transporter family together with a topology model. To evaluate the involvement of the conserved C-terminal part of the CitP and MleP transporters in substrate binding, four chimeric transporters (CM397, CM353, MC397 and MC353) were constructed. Capitals indicate the origin of the N- and C-terminal parts of the chimera (C for CitP, M for MleP) and the number indicates the position of the amino acid residue preceding the junction (CitP numbering). Thus, CM397 consists of the 397 N-terminal residues of CitP and the 46 C-terminal residues of MleP, whereas the complementary chimera MC397 consists of the corresponding N-terminal part of MleP followed by the C-terminal residues of CitP. Wild type and chimeric transporters were expressed in *L. lactis* NZ9000 cells under control of the inducible *nisA* promoter (12). The expression vectors differed only in the coding sequences of the transporter proteins. Wild type and chimeric transporters were tagged with 10 histidine residues at the N-terminus to allow analysis of expression levels (see Experimental Procedures).

Expression of wild-type and chimeric transporters. The expression levels of wild-type and chimeric transport proteins in *L. lactis* NZ9000 cells were analyzed by Western blotting using antibodies raised against the His-tag (Figure 3). MleP runs as a protein with an apparent molecular mass of around 35 kDa, while CitP runs at approximately 40 kDa (lanes 1 and 4). The level of expression of MleP was significantly higher than observed for CitP. The apparent molecular masses of the chimeras correlated with the transporter that donated the N-terminal part of the sequence, i.e., the residues responsible for the different mobility of CitP and MleP

Table 1. **Affinity constants of wild-type and chimeric transporters for external substrates.**

	CitP		CM397		MleP		MC397	
	K_i (mM)	K_m (mM)	K_i (mM)	K_m (mM)	K_i (mM)	K_m (mM)	K_i (mM)	K_m (mM)
(S)-malate	0.068 ± 0.009	0.10 ± 0.022	0.056 ± 0.015	0.091 ± 0.022	–	0.47 ± 0.1	–	0.64 ± 0.09
citrate	0.056 ± 0.009[a]	–	0.042 ± 0.009	–	9 ± 2[a]	–	8.5 ± 2	–
(S)-mandelate	>100[b]	–	63 ± 5	–	3.5 ± 0.5	–	13.5 ± 2	–
(S)-2-hiv[c]	>150[b]	–	28 ± 4	–	28 ± 4	–	>350[b]	–
(R)-mandelate	–	7 ± 2	–	5 ± 3	4.0 ± 0.8	–	4.7 ± 0.6	–
(R)-2-hiv[c]	–	12.5 ± 3	–	3.1 ± 1.9	29 ± 9	–	102 ± 26	–

[a]Ki value taken from reference (8). [b]Lower limit of Ki values estimated from the inhibition at a external substrate concentration of 20 mM. [c](S)-2-hiv, (S)-2-hydroxyisovalerate. (R)-2-hiv, (R)-2-hydroxyisovalerate.

on SDS-PAGE are located in the 353 N-terminal residues. The level of expression of the chimeras was considerably less than observed for the wild-type proteins and dropped dramatically when the junction was more towards the N-terminus in CM353 and MC353. Again, expression levels of the chimeras seem to be related to the N-terminal sequences since expression of the MC chimeras was higher than observed for the corresponding CM chimeras (compare lanes 2 and 3 and lanes 5 and 6, respectively).

(S)-Malate transport activity. (S)-Malate is a high affinity substrate of both CitP and MleP (3). The chimeric transporters were assayed for their ability to catalyze homologous (S)-malate counterflow and equilibrium exchange. In counterflow, right-side-out membranes prepared from cells expressing the wild-type or chimeric proteins were loaded with 5 mM (S)-malate, and subsequently, diluted 100-fold into buffer containing 9.8 μM ^{14}C labelled (S)-malate. The gradients of labelled and unlabeled (S)-malate over the membrane drive the label into the lumen of the vesicles via exchange catalyzed by the transporters (3). The initial rates of counterflow in membranes containing wild-type CitP and MleP were very similar (Figure 4A, closed and open circles). Since the membranes used in these experiments were the same as those used for the immunoblots shown in Figure 3, it follows that the specific activity of MleP is lower than the specific activity of CitP under these conditions. The initial rate of counterflow of the MC397 chimeric transporter was somewhat lower than observed for the wild-type proteins, while the complementary CM397 transporter exhibited at least a 10-fold lower activity. These reductions correlate with the reduced level of expression of the chimeric proteins when compared to MleP and CitP. No significant counterflow activity was observed for the MC353 and CM353 proteins.

Similar observation where made for equilibrium exchange (Figure 4B). In equilibrium exchange, right-side-out membranes were loaded with 5 mM ^{14}C-labeled (S)-malate and, subsequently, diluted 100-fold in buffer containing the same concentration of unlabeled (S)-malate. Exchange catalyzed by the transporters results in depletion of the radio label in the lumen of the vesicles. CitP and MleP and the MC397 chimeric protein exhibited very high and similar exchange rates, while the exchange rate of the CM397 transporter was significantly lower. No exchange activity could be detected for the MC353 and CM353 proteins.

The affinity of the transporters for external (S)-malate was determined by measuring the exchange and/or counterflow activity at a range of external (S)-malate concentrations. Since no exchange or counterflow activity was observed for CM353 and MC353 these chimeras were excluded from further studies. Under standard expression conditions, exchange and counterflow were too fast to measure initial rates in membranes containing CitP, MleP and MC397 (see Figure 4). To allow kinetic analysis of these transporters, expression levels and thus the initial rates were reduced by manipulating the inducer concentration in the growth medium and the induction time. Affinity constants for external (S)-malate were found to be 0.47 mM and 0.084 mM for MleP and CitP, respectively (Table 1), similar to what has been

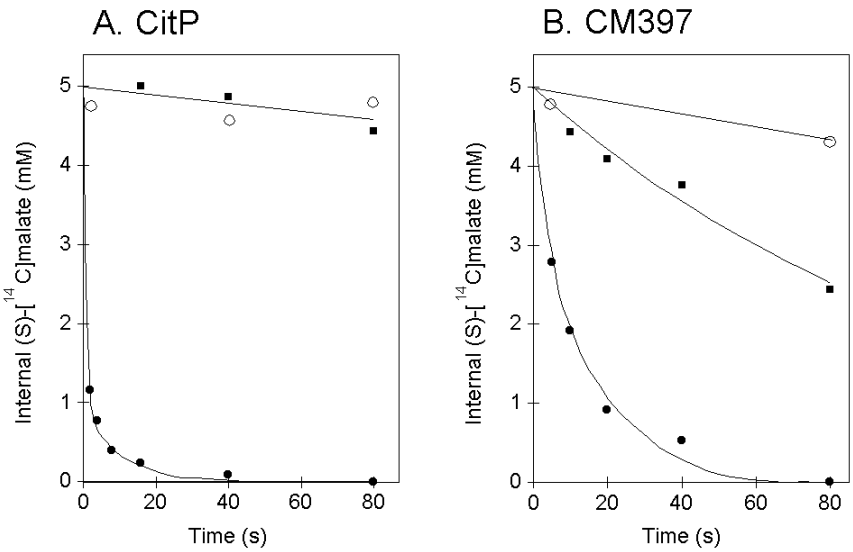

Figure 5. **(S)-Mandelate-(S)-malate exchange catalyzed by CitP (A) and CM397 (B).** RSO membrane vesicles of *L. lactis* NZ9000 expressing CitP (A) and CM397 (B) were preloaded with 5 mM (S)-[^{14}C]malate and diluted 100-fold into buffer containing no further additions (open circles), 5 mM (S)-malate (closed circles), and 5 mM (S)-mandelate (closed squares).

reported before for CitP and MleP without the N-terminal His-tags (8). The affinity of CM397 for (S)-malate was not significantly different from the affinity of CitP, and similarly, the affinity of MC397 was very close to the affinity of MleP. In conclusion, the activities of the chimeric proteins with (S)-malate as the substrate correlate with the levels of expression of the proteins, and the affinities of the CM397 and MC397 chimeras appear to be determined by the N-terminal parts of the chimeras.

Specificity for citrate. CitP translocates citrate with high efficiency, while MleP is unable to translocate citrate, but still binds citrate with low affinity (8). The affinity of the transporters for external citrate was measured by substituting (S)-malate in the dilution buffer in the exchange assay for citrate (heterologous exchange) or by assessing the inhibition of (S)-malate counterflow by external citrate. When corrected for the external (S)-malate concentration the affinity constant for heterologous exchange and the inhibition constant for counterflow represent the same kinetic parameter (for a discussion, see ref 8). The affinity constant of CitP for external citrate in the heterologous exchange reaction was 0.056 mM, while the inhibition constant of citrate for MleP catalyzed counterflow was 9 mM (Table 1). Chimera CM397 effectively exchanged (S)-malate for citrate with an affinity constant for external citrate that was similar as observed for CitP (Table 1). The complementary chimera MC397 was unable to translocate citrate (not shown), but citrate inhibited (S)-malate counterflow with an inhibition constant of 8.7 mM as was observed for MleP. In conclusion, the structural elements enabling CitP to translocate citrate seem to be located outside the C-terminal 46 amino acids.

Specificity for monocarboxylates. The results described above indicate that interchanging the 46 C-terminal amino acid residues of CitP and MleP altered neither the specificity nor the affinity for the di- and tricarboxylates malate and citrate. In contrast, changes in substrate specificity were observed with monocarboxylates. Mandelate is a 2-hydroxycarboxylate in which the two R groups are a hydrogen atom and a phenyl group (Figure 1). (S)-mandelate at a concentration of 5 mM did not induce release of ^{14}C labeled (S)-malate from right-side-out membranes containing CitP, while exchange with 5 mM (S)-malate was fast in the same membranes (Figure 5A). At 20 mM (S)-mandelate, a low but significant exchange was observed, indicating that CitP could translocate (S)-mandelate (not shown). Replacement of the 46 C-terminal amino acids with the corresponding MleP residues enabled the resulting CM397 chimera to catalyze heterologous (S)-mandelate-(S)-malate exchange at a significant rate at 5 mM (S)-mandelate (Figure 5B). Homologues (S)-malate exchange rate was reduced due to the lower level of expression of the chimeric transporter. Clearly, the substrate selectivity of the

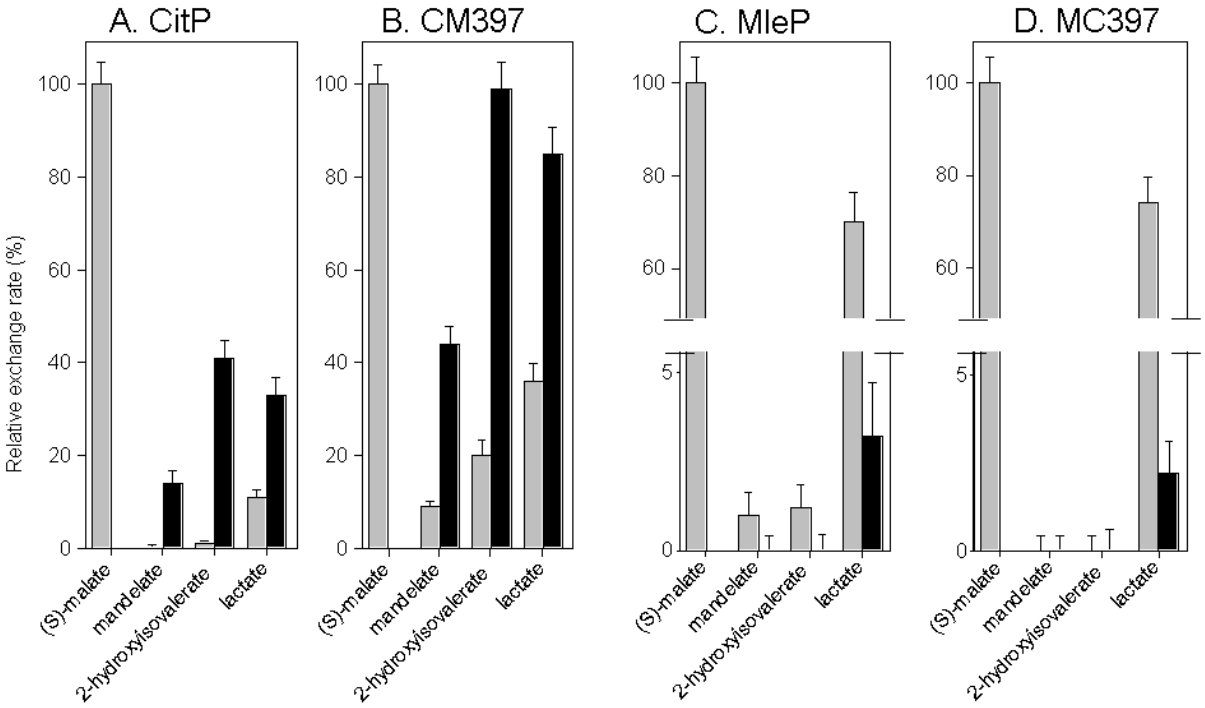

Figure 6. **Heterologous exchange catalyzed by the wild-type and chimeric transporters.** RSO membrane vesicles of *L. lactis* NZ9000 expressing CitP (A), CM397 (B), MleP (C), and MC397 (D) were preloaded with 5 mM (S)-[^{14}C]malate and diluted 100-fold into buffer containing the indicated substrates at 5mM. Left (gray) and right (black) bars correspond to the (S)- and (R)-enantiomeric forms of the substrates, respectively. Rates represent the average of 2 to 4 independent measurements and are given relative to the rate observed for homologous (S)-malate exchange. Error bars indicate the standard deviations.

chimeric transporter had changed. Under the same conditions, exchange rates for a number of other monocarboxylates, i.e. (R)-mandelate, (S)- and (R)- 2-hydroxyisovalerate, (S)- and (R)-lactate were determined for CitP and CM397. The result showed a similar increase in the relative exchange rates (Figure 6A,B).

Except for (S)- and (R)-lactate, heterologous exchange with the same compounds catalyzed by MleP was much slower and significant only for the S-enantiomers of mandelate and 2-hydroxyisovalerate (Figure 6C,D). Heterologous lactate exchange was similar as observed for MleP without the His-tag (8). Introducing the 46 C-terminal residues of CitP in MleP yielding MC397 resulted in the complete loss of exchange activity with these compounds, i.e., the effect was reciprocal to what was observed with the CitP and CM397 transporters. No change in specificity for (S)- and (R)-lactate was observed for the MleP and MC397 transporters.

The changes in heterologous exchange activity of the chimeric proteins observed with the monocarboxylates reported in Figure 6 do not discriminate between changes in affinity and changes in translocation efficiency. The affinities of the transporters for the substrates were measured as discussed above for citrate. The low rate of exchange observed for (S)-mandelate and (S)-2-hydroxyisovalerate catalyzed by CitP was due to low affinity of the transporter for these substrates. In fact, the affinity constants were too high to be measured accurately and were estimated to be over 100 mM for (S)-mandelate and even over 150 mM for (S)-2-hydroxyisovalerate (Table 1). In contrast, the low exchange rates with these compounds catalyzed by MleP were due to the lack of translocation; the inhibition constants for (S)-mandelate and (S)-2-hydroxyisovalerate were 3.5 and 31 mM, respectively. These results reveal an important difference between CitP and MleP; MleP binds the S-enantiomers of these two substrates with much higher affinity than CitP, but CitP translocates them better than MleP. A similar observation was reported before for the physiological substrate lactate (8). The R-enantiomers of mandelate and 2-hydroxyisovalerate bound with similar affinities to CitP and MleP.

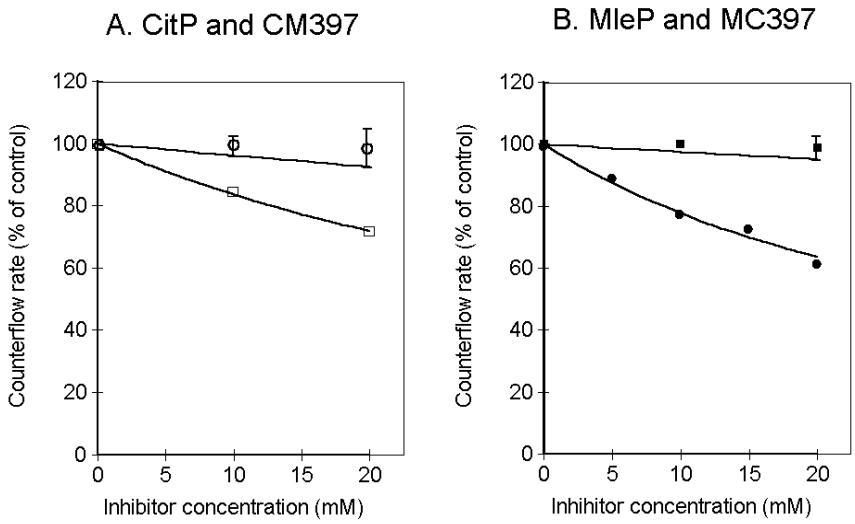

Figure 7. **Inhibition of (S)-malate counterflow by (S)-2-hydroxyisovalerate.** RSO membrane vesicles of *L. lactis* NZ9000 expressing (A) CitP (open circles) and CM397 (open squares) and (B) MleP (closed circles) and MC397 (closed squares) were loaded with 5 mM (S)-malate and diluted 100-fold in buffer containing 9.8 μM (S)-[^{14}C]malate and increasing concentrations of (S)-2-hydroxyisovalerate. Rates were indicated as the percentage of the rate observed in the absence of inhibitor. The deduced K_i values corrected for the external (S)-malate concentration (59.8 μM) are given in Table 1.

The improved exchange observed for (S)-mandelate and (S)-2-hydroxyisovalerate when the 46 C-terminal residues of CitP were replaced by the corresponding residues of MleP was caused by an improved affinity of the CM397 chimera for these substrates (Table 1). The C-terminal residues of MleP seem to introduce the higher affinity of MleP for these substrates into the chimera. Most importantly, the affinity of the complementary chimera MC397 for these substrates decreased relative to the affinities of MleP for these substrates. The largest affinity changes were observed for (S)-2-hydroxyisovalerate for which the affinity increased by a factor of at least 5 between CitP and CM397 and decreased by a factor of at least 10 between MleP and MC397 (Figure 7). The chimeras seem to adopt the affinity for the S-enantiomers of mandelate and 2-hydroxyisovalerate of the transporter that donated the C-terminus.

The observed changes in affinity between the parent and chimeric proteins were only minor for the R-enantiomer of mandelate indicating that the higher heterologous exchange rate observed for CM397 relative to CitP (Figure 6A,B) was mainly due to an improved translocation efficiency. In contrast, the affinities for the R-enantiomer of 2-hydroxyisovalerate changed 3-fold and in the opposite direction for CM397 and MC397. Replacing the C-terminal residues in CitP resulted in a increase in affinity, while replacing the same residues in MleP resulted in a decrease in affinity (Table 1).

DISCUSSION

The malate transporter MleP and the citrate transporter CitP are homologous proteins with 48% identical amino acid residues in their primary structure. Both proteins transport structurally related substrates and differences in substrate specificity between MleP and CitP were found to be due to subtle, but well defined, differences in binding and/or translocation properties (8). The conserved nature of the substrate binding site led us to believe that structural elements involved in substrate recognition might be located in conserved stretches of the amino acid residues. Highest level of sequence conservation in the 2-HCT family is found in a stretch of 46 residues at the C-terminus. To analyze the involvement of the C-terminal region in substrate recognition we have interchanged the C-terminal stretches between the two transporters and analyzed the resulting chimeras for substrate specificity. An advantage of this approach is that the subtle differences in substrate specificity between CitP and MleP may

depend on a combination of different amino acid residues at multiple positions which may be difficult to trace using site directed mutagenesis.

Activities of the CM397 and MC397 chimeric transporters with (S)-malate as the substrate were lower than observed for the wild type transporters. The activities correlated with the reduced levels of expression of the chimeric proteins when CM397 was compared to CitP and MC397 to MleP, suggesting that the specific (S)-malate transport activity remained unchanged upon replacement of the C-terminal sequences. The loss of expression was dramatic when the fusion point was moved further to the N-terminus in chimeras CM353 and MC353 (Figure 3) for which no exchange activity could be detected. Similar observations were made with chimeras between CitP and MleP that had fusion points at positions 256 and 298 (not shown). The lower expression levels of CM397 and MC397 and the other chimeras seem unlikely to be due to a loss of structural integrity and, consequently, degradation of the chimeras. The chimeric molecules that enter the plasma membrane had the same kinetic parameters as the parental proteins (see below) and no breakdown products could be detected in either membrane vesicles or whole cells expressing the chimeras (not shown). Interestingly, expression levels of the wild type transporters were also found to differ greatly even though expression system, and induction and growth conditions were exactly the same.

CitP and MleP transport substrates that present the 2-hydroxycarboxylate motif (HO-CR_2-COO^-). Previous studies of the catalytic properties of the two transporters with a large range of 2-hydroxycarboxylates (3,8) have resulted in a model of the substrate binding site that indicates the interaction between the functional groups of the substrates and (unknown) groups on the proteins (Figure 1). In the model, two essential interaction are between groups in the binding pocket and the carboxylate and hydroxyl groups of the 2-hydroxycarboxylate motif that is common to all substrates. These interactions are like anchors that orient the substrates in the binding pocket. A third, non-essential anchoring point in the pocket (R_S side) interacts with a second carboxylate group on the substrates. This interaction is responsible for high affinity binding of di- and tricarboxylates with a second carboxylate at R_S. (S)Malate interacts with the protein via these three interactions. The kinetic parameters of the chimeric proteins CM397 and MC397 with (S)-malate as the substrate were the same as observed for the parental transporters that donated the N-terminal part, indicating that the spatial arrangement of the three anchoring points on the proteins was the same as in the parental proteins. The most straight forward conclusion would be that the entire binding pocket would be located in the N-terminal 397 residues, however, the possibility that part part of the interacting sites in the binding pocket are conserved in the 46 C-terminal residues cannot be excluded. Clearly, the difference in affinity of CitP and MleP for (S)-malate is coded in the N-terminal part.

The citrate molecule differs from the (S)-malate molecule in that it has a CH_2COOH group instead of a H-atom at the R_R position. This carboxylate group is protonated when the transporters interact with citrate (8). The CitP protein has no problem to accommodate the group and binds and transports citrate as efficient as it does (S)-malate. On the other hand, MleP has at least a 10-fold lower affinity for citrate as compared to (S)-malate and is unable to translocate citrate. In this respect, the MC397 chimera behaved exactly like MleP and the CM397 chimera like CitP, indicating that the structural elements interacting with the CH_2COOH group of citrate at the R_R side of the binding pocket are located in the N-terminal part of the proteins.

The catalytic properties of CitP and MleP with citrate as the substrate were also recognised in the activities with monocarboxylates with a hydrophobic group at the R_R side like (R)-mandelate and (R)-hydroxyisovalerate. Both transporters exhibited affinity for the substrates, but as was observed for citrate, MleP was found to be unable to transport them (Table 1; 8). However, unlike with citrate, replacement of the 46 C-terminal residues in the two chimeras did affect the catalytic properties with the R-enantiomers of the monocarboxylates. Introduction of the C-terminus of MleP in CitP yielding CM397 resulted in a higher exchange activity relative to (S)-malate (Figure 6). Kinetic analysis revealed that the improved activity

with (R)-mandelate was caused by an improved translocation efficiency of CM397 relative to CitP, while in the case of (R)-2-hydroxyisovalerate, the affinity of the chimera improved 4-fold (Table 1). The different mechanisms suggest a different interaction of the two hydrophobic R-groups with the proteins. Importantly, the affinity for (R)-2-hydroxyisovalerate decreased between MleP and MC397 by a factor of 3-4, showing that the results of interchanging the C-terminal sequences were reciprocal. A reciprocal effect on the translocation efficiency of (R)-mandelate was undetectable since MleP showed no activity with (R)-mandelate to begin with. In conclusion, even though the effects of interchanging the C-terminal residues on the R_R groups of the substrates are not specific, and as such, do not provide evidence for a direct interaction between the C-terminal residues and the R_R groups of the substrates, the results do indicate the involvement of the C-terminal region in catalysis.

The strongest evidence that the 46 C-terminal residues in CitP and MleP constitute part of the substrate binding pocket comes from the affinity changes observed for the S-enantiomers of the monocarboxylates mandelate and 2-hydroxyisovalerate that interact with the R_S side of the binding pocket. CitP has very little affinity for these substrates, but translocates them with high efficiency. In contrast, MleP binds these substrates with an affinity that is at least an order of magnitude higher, but translocation is very inefficient. The chimeras CM397 and MC397 adopted specifically the affinities, not the translocation efficiencies, for (S)-mandelate and (S)-hydroxyisovalerate from the parent that donated the C-terminal residues (Table 1 and Figures 6 and 7). Apparently, the C-terminal residues are involved in the initial recognition of the substrates. Consequently, at 5 mM concentrations, heterologous exchange activity with these substrates improves considerable in CM397 and is diminished in the complementary chimera MC397. Arguments against long range conformational effects and in favour of a model in which the 46 C-terminal residues contain the structural elements at the R_S side of the binding pocket are (i) the effect in the two chimeras are reciprocal, (ii) the chimeras adopt a property of the donor of the C-terminal sequences, and (iii) the C-terminal sequences specifically effect the affinity for the substrates.

The model for the membrane topology of CitP and MleP shows 11 putative transmembrane α-helices with the N-terminus in the cytoplasm and the C-terminus in the periplasm. The C-terminal region that in this study was interchanged between the two transporters consists of TMS XI that is outgoing and part of the cytoplasmic loop between TMSs X and XI. The present study suggests that the C-terminal region interacts directly with the hydrophobic R_S-groups of the monocarboxylate substrates, and thus suggests that the binding site is at the interface of TMS XI and the other TMSs of the proteins. The hydrophobic groups at the R_S side of the binding pocket seem to interact with similar sites located on TMS XI since exchange of the C-terminal parts affects the kinetics with different substrates in a similar manner. The R_R groups of the substrates that are necessarily close in the binding pocket, seem to have a less well-defined interaction with the proteins. In citrate, the polar CH_2COOH group interacts with the N-terminal TMSs while the hydrophobic R-groups of (R)-2-hydroxyisovalerate and (R)-mandelate sense changes in the C-terminus, but not in the same way. This interaction of functional groups located in space at the same side of a substrate molecule with different parts of the protein is reminiscent of what has been proposed to be the mechanism by which multidrug transporter proteins interact with their substrates (20-22). No change in the three anchoring points that interact with the hydroxyl and carboxylate group of the substrate motif and the second carboxylate at R_S was observed in the chimeras, indicating that there relative localization in the binding pocket is unchanged. The anchoring points are either in the N-terminal part or conserved between CitP and MleP in the 46 C-terminal residues. The C-terminal part contains two conserved arginines, one of which is located in the TMS XI, that are good candidates to be involved in the electrostatic interactions with the carboxylate groups of the substrates. At present, we are mutating these and other residues to further analyze the role of TMS XI in substrate recognition.

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REFERENCES

1. Marty Teysset, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1995) *J. Biol. Chem. 270*, 25370-25376.
2. Bandell, M., Lhotte, M. E., Marty Teysset, C., Veyrat, A., Prevost, H., Dartois, V., Divies, C., Konings, W. N., and Lolkema, J. S. (1998) *Appl. Environ. Microbiol. 64*, 1594-1600.
3. Bandell, M., Ansanay, V., Rachidi, N., Dequin, S., and Lolkema, J. S. (1997) *J. Biol. Chem. 272*, 18140-18146.
4. Poolman, B., Molenaar, D., Smid, E. J., Ubbink, T., Abee, T., Renault, P. P., and Konings, W. N. (1991) *J. Bacteriol. 173*, 6030-6037.
5. Lolkema, J. S., Poolman, B., and Konings, W. N. (1996) in *Handbook of Biological Physics* (Konings, W. N., Kaback, H. R., and Lolkema, J. S., Eds.) pp 229-260, Elsevier, Amsterdam.
6. Marty Teysset, C., Posthuma, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1996) *J. Bacteriol. 178*, 2178-2185.
7. Magni, C., de Mendoza, D., Konings, W. N., and Lolkema, J. S. (1999) *J. Bacteriol. 181*.
8. Bandell, M. and Lolkema, J. S. (1999) *Biochemistry 38*, 10352-10360.
9. van Geest, M. and Lolkema, J.S. (1999) *J. Biol. Chem. 274*, 29705-29711.
10. van Geest, M., Nilsson, I., von Heijne, G., and Lolkema, J. S. (1999) *J. Biol. Chem. 274*, 2816-2823.
11. van Geest, M. and Lolkema, J.S. (2000) *Biochim. Biophys. Acta 1466,* 328-338.
12. de Ruyter, P. G., Kuipers, O. P., and De Vos, W. M. (1996) *Appl. Environ. Microbiol. 62*, 3662-3667.
13. Kuipers, O. P., Beerthuyzen, M. M., Siezen, R. J., and De Vos, W. M. (1993) *Eur. J. Biochem. 216*, 281-291.
14. Putman, M., van Veen, H. W., Poolman, B., and Konings, W. N. (1999) *Biochemistry 38*, 1002-1008.
15. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
16. Holo, H. and Nes, I. F. (1989) *Appl. Environ. Microbiol. 55*, 3119-3123.
17. Gaillard, I., Slotboom, D. J., Knol, J., Lolkema, J. S., and Konings, W. N. (1996) *Biochemistry 35*, 6150-6156.
18. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) *Gene 77*, 61-68.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
20. Zheleznova, E. E., Markham, I., Edgar, I., Bibi, I., Neyfakh, A. A., and Brennan, R. G. (2000) *Trends Biochem. Sci. 25*, 39-43.
21. Zheleznova, E. E., Markham, P. N., Neyfakh, A. A., and Brennan, R. G. (1999) *Cell 96,* 353-362.
22. Vazquez-Laslop, N., Markham, P. N., and Neyfakh, A. A. (1999) *Biochemistry 38*, 16925-16931.
23. van der Rest, M. E., Siewe, R. M., Abee, T., Schwarz, E., Oesterhelt, D., and Konings, W. N. (1992) *J. Biol. Chem. 267*, 8971-8976.
24. Kawai, S., Suzuki, H., Yamamoto, K., and Kumagai, H. (1997) *J. Bacteriol. 179*, 4056-4060.
25. Kunst, F., Ogasawara, N., Moszer, I., and 148 other authors (1997) *Nature 390*, 249-256.

Chapter 6

Arg425 of the Citrate Transporter CitP is Responsible for High Affinity Binding of Di- and Tricarboxylates

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SUMMARY

The citrate transporter of *Leuconostoc mesenteroides* (CitP) catalyzes exchange of divalent anionic citrate from the medium for monovalent anionic lactate, which is an end product of citrate degradation. The exchange generates a membrane potential, and thus metabolic energy for the cell. The mechanism by which CitP transports both a divalent and a monovalent substrate was the subject of this investigation. Previous studies indicated that CitP is specific for substrates containing a 2-hydroxycarboxylate motif, $HO\text{-}CR_2\text{-}COO^-$. CitP has a high affinity for substrates that have a "second" carboxylate at one of the R groups, such as divalent citrate and (S)-malate (6). Monovalent anionic substrates that lack this "second" carboxylate were found to bind with a low affinity. In the present study we have constructed site directed mutants, changing Arg425 into a lysine or a cysteine residue. Using two substrates, i.e. (S)-malate and 2-hydroxyisobutyrate, the substrate specificity of the mutants was analyzed. In both mutants the affinity for divalent (S)-malate was strongly decreased while the affinity for monovalent 2-hydroxyisobutyrate was not. The largest effect was seen when the arginine was changed into the neutral cysteine, which reduced the affinity for (S)-malate over 50 fold. Chemical modification of the Arg425Cys mutant with the sulfhydryl reagent 2-aminoethyl methanethiosulfonate which restores the positive charge at position 425 dramatically reactivated the mutant transporter. The Arg425Cys and Arg425Lys mutants revealed a substrate protectable inhibition by other sulfhydryl reagents and the lysine reagent 2,4,6-trinitrobenzene sulfonate, respectively. It is concluded that Arg425 complexes the charged carboxylate present in divalent substrates but absent in monovalent substrates, and thus plays an important role in the generation of the membrane potential.

INTRODUCTION

In recent years a growing number of secondary transporters have been discovered that generate rather than consume metabolic energy. These transporters have been termed precursor-product exchangers since they catalyze the uptake of a substrate into the cell coupled to the exit of a metabolic end product into the medium (for a review see ref. 1). An example of such a transporter is the citrate transporter (CitP) found in *Leuconostoc mesenteroides* (2,3) which catalyzes the uptake of divalent citrate into the cell coupled to the exit of monovalent lactate, a metabolic endproduct of citrate degradation in lactic acid bacteria (4). The net charge movement over the membrane during the exchange results in a membrane potential of physiological polarity. This, in combination with the consumption of a cytoplasmic proton in the breakdown of citrate results in a proton motive force, and thus generates metabolic energy

for the cell. Recovery from acidic stress and resistance against lactate toxicity have recently been suggested as alternative roles for the metabolic pathway (5).

The ability of CitP to transport two such different substrates as citrate and lactate is associated with a high specificity for the 2-hydroxycarboxylate motif present in both substrates (i.e. $HO\text{-}CR_2\text{-}COO^-$), in combination with a high promiscuity towards the two R groups (6). In fact, next to the physiological substrates, CitP is able to transport a wide range of 2-hydroxycarboxylates containing various R groups. The ability to accept both a neutral as well as a negatively charged R group is the basis for membrane potential generation by CitP. CitP is known to have a high affinity for divalent di- and tricarboxylates like citrate and (S)-malate, that have a "second" carboxylate at one of the R groups (6). Monocarboxylates like lactate and 2-hydroxyisobutyrate (2-HIB), that lack this "second" carboxylate were found to bind with a low affinity but were still transported efficiently. The high affinity of CitP for substrates with a "second" carboxylate was explained by postulating a strong, possibly electrostatic, interaction between the protein and the "second" carboxylate. Positively charged residues such as arginine, lysine or histidine were thought to be able to participate in such an interaction.

CitP belongs to the 2-hydroxycarboxylate transporter (2-HCT) family (7), that contains members found in several lactic acid bacteria as well as in *Bacillus subtilis* and *Klebsiella pneumoniae* (8). The family contains precursor-product exchangers like CitP and the malate transporter MleP of *Lactococcus lactis*, but also the Na^+/citrate symporter CitS of *K. pneumoniae*, suggesting that precursor-product exchangers are "normal" secondary transporters that have been optimized to catalyze exchange. Transporters of the 2-HCT family are believed to consist of 11 transmembrane segments (TMSs), based on topology studies of CitS of *K. pneumoniae* (9-11). The C-terminus of the proteins resides in the periplasm. A recent study of chimeras between the citrate transporter CitP and the malate transporter MleP indicated that the C-terminal region including TMS XI forms part of the substrate binding site that interacts with the R-groups of the substrates (8). The C-terminal region contains two conserved arginine residues one of which is located in TMS XI. The latter residue was thought to be a good candidate for an interaction with the "second" carboxylate of divalent substrates.

In the present study Arg425 in TMS XI of CitP was replaced by lysine and cysteine residues. The mutant transporters were analyzed for their affinity towards the divalent and monovalent substrates (S)-malate and 2-HIB, respectively. In addition, the effect of chemical modification of residues at position 425 and the ability of substrate to protect against modification was investigated. It is concluded that Arg425 is located in the substrate binding site where it is responsible for a high affinity interaction with the "second" carboxylate of di- and tricarboxylates.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. *L. lactis* strain NZ9000 is a MG1363 derivative (*pepN*::*nisRnisK*; (12)) that transports citrate nor malate. The *nisR* and *nisK* genes were inserted in the chromosome to allow induced expression of plasmid encoded genes under control of the tightly regulated *nisA* promoter (13). Cells harbouring the expression vector pNZcitP containing the *citP* gene (see below) were grown at 30 °C in closed serum bottles and without shaking in M17 broth (Difco) supplemented with 0.5% (w/v) glucose and 5 μg/mL chloramphenicol. Unless otherwise stated, expression of the transporter was induced by growing the cells to an optical density of 0.6 measured at 660 nm (OD_{660}), followed by addition of a 1000-fold dilution of the supernatant of an over night culture of the nisin producing *L. lactis* strain NZ9700 into the cultures (14). The supernatant contained approximately 10 ng of nisinA/mL (15). Growth was continued for 1 h followed by harvesting of the cells by centrifugation. In some cases a lower level of induction was required to allow kinetic analysis (see Results). Then, cells expressing CitP were grown in the presence of a 30,000-fold dilution of the nisin containing supernatant for 0.5 h.

DNA manipulations. General procedures for cloning and DNA manipulations were performed essentially as described by Sambrook et al. (16). Expression vector pNZcitP codes for the CitP protein with 10 additional histidine residues at the N-terminus (8). Arg425Cys and Arg425Lys mutations were introduced in the *citP* gene in two steps by overlap extension PCR (17). All PCR-amplified DNA fragments were sequenced to confirm the nucleotide sequence. Ligation mixtures were transformed to *L. lactis* NZ9000 by electroporation as described by Holo and Nes (18).

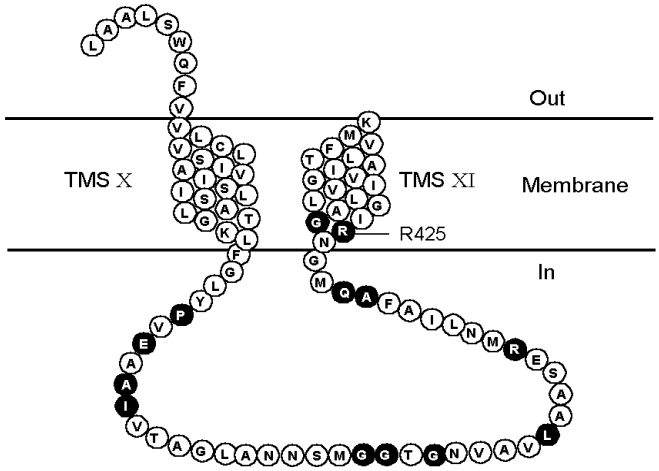

Figure 1. **Membrane topology and amino acid sequence of the C-terminal part of CitP.** The topology model is based on studies of the Na^+-dependent citrate transporter CitS of *K. pneumoniae* which is homologous to CitP (11). Conserved residues in the amino acid sequence alignment of the 2-HCT family (8) were indicated in gray.

Preparation of right-side-out membrane vesicles. Right-side-out (RSO) membrane vesicles of NZ9000 cells expressing wild type or mutant CitP were prepared by the osmotic shock lysis procedure in the presence of 5 mM (S)-malate, essentially as described previously (6). Membrane vesicles were washed ones with 50 mM potassium phosphate pH 6, containing 5 mM (S)-malate and concentrated by centrifugation in an Eppendorf table top centrifuge operated at full speed for 5 min. The internal pool of (S)-malate was labelled with (S)-[^{14}C]malate by incubating the concentrated membranes with 186.7 µM of L-[1,4(2,3)^{14}C]malate for 1 h in the presence of 1 mM valinomycin and 0.5 mM nigericin. Protein concentrations were determined as described by Lowry et al. (19). In case the vesicles were used for chemical modification studies, (S)-malate was omitted in the preparation procedure and loading of the vesicles with (S)-[^{14}C]malate was done as described under "Chemical modification".

Exchange measurements. Aliquots of 2 µL (S)-[^{14}C]malate loaded vesicles were diluted into 200 µL of 50 mM potassium phosphate pH 6, containing substrates at the indicated concentrations at 20 °C. Internal radioactivity was determined at different time points by dilution with ice cold LiCl followed by rapid filtration, as described (6). Final membrane protein concentrations in the assays were between 250 and 350 µg/mL. To evaluate the data, initial rates of exchange were determined by fitting the data to an exponential decay using non-linear fitting procedures provided by the Sigma Plot software (Jandel Scientific, San Rafael, CA), as described previously (7). The rate of efflux in the absence of external substrate was subtracted from the observed rates. Exchange rates were determined at different external substrate concentrations to estimate the affinity constant K_m^{app}. The data was fitted to an equation describing competitive inhibition taking into account the effect of (S)-[^{14}C]malate in the external buffer caused by the dilution of the (S)-[^{14}C]malate loaded membrane vesicles (6).

Chemical modification. RSO membrane vesicles (10 mg protein/ml) prepared in the absence of (S)-malate were incubated for the indicated times in 50 mM potassium phosphate containing 5 mM of the lysine specific reagent TNBS (Fluca) or 1 mM of the sulfhydral reagents MTSEA, MTSES, MTSET (Anatrace), PCMB and PCMBS (Sigma) at 23 °C. Reactions with the sulfhydral reagents were performed at pH 6, and with TNBS at a range of pH values. Following incubation, the vesicles were diluted 15 times in 50 mM potassium phosphate pH 6, and concentrated by centrifugation in an Eppendorf table top centrifuge operated at full speed for 5 min. The membranes were washed 3 times using the same procedure. To load the vesicles with (S)-[^{14}C]malate, the concentrated vesicles were incubated overnight at 4 °C in the presence of 5 mM (S)-malate, 186.7 µM L-[1,4(2,3)^{14}C]malate, 1 mM valinomycin and 0.5 mM nigericin. Exchange activity was determined as described above.

To test the effect of substrate on chemical modification, RSO membranes were preincubated for 2 h with and without the substrate in the presence of 1 mM valinomycin and 0.5 mM nigericin followed by reaction with 0.1 mM PCMB for 0.5 min or with 30 mM TNBS for 2 h at pH 7. The membranes were washed twice with 50 mM potassium phosphate pH 6, in the presence of substrate and 4 times in the absence of substrate. The concentrated vesicles were loaded with 5 mM radiolabeled (S)-malate and exchange activity was determined as described above. To evaluate the efficiency of the washing procedure for removing internal substrate, vesicles were equilibrated with 60 mM 2-HIB, butyrate, (S)-malate, (S)-citramalate or succinate and were washed four times. In all cases exchange rates were not significantly different from vesicles that were incubated in the absence of substrate (data not shown).

SDS-PAGE and immunoblot analysis. Right side out membrane vesicles were subjected to SDS-PAGE using a 12% polyacrylamide gel matrix (15 µg protein/lane). After electrophoresis, the proteins were transferred to poly(vinylidenedifluoride) membranes and analyzed using monoclonal antibodies directed against a His-tag (Dianova, Hamburg, Germany). Antibodies were visualised using the Western-light chemiluminesence detection kit (Tropix, Bedford, MA)

Chemicals. L-[1,4(2,3)-^{14}C]malic acid (51 mCi/mmol) was obtained from Amersham International (Buckinghamshire, U.K.). All other compounds were obtained from Fluca (Buchs, Switzerland) or Sigma (St. Louis, MO, USA).

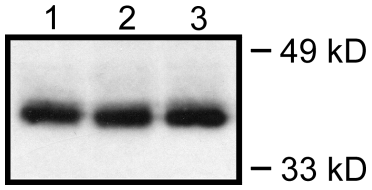

Figure 2. **Expression levels of CitP, Arg425Lys and Arg425Cys.** RSO membrane vesicles prepared of *L. lactis* NZ9000 expressing CitP (lane 1), Arg425Lys (lane 2) and Arg425Cys (lane 3) were analyzed by western blotting using antibodies raised against the His-tag. The position of the molecular weight markers was indicated on the right.

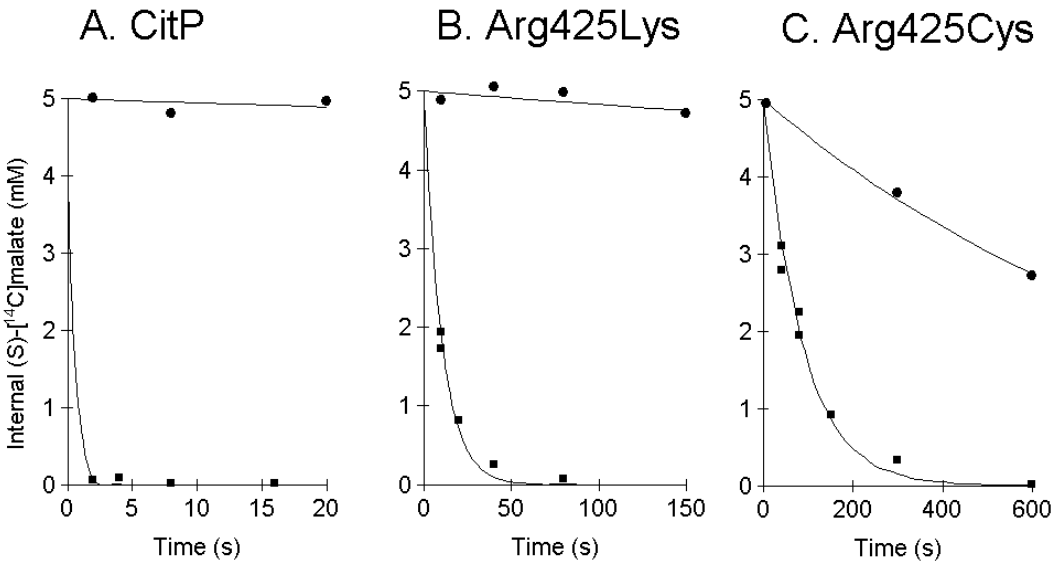

Figure 3. **Homologous (S)-malate exchange catalyzed by CitP, Arg425Lys and Arg425Cys.** RSO membrane vesicles prepared of *L. lactis* NZ9000 expressing CitP (A), Arg425Lys (B) and Arg425Cys (C) were preloaded with 5 mM (S)-[^{14}C]malate and diluted 100 fold into buffer containing 5 mM (S)-malate (closed squares) or no additions (closed circles).

RESULTS

Construction and activity of the Arg425Cys and Arg425Lys mutant transporters. Arg425 is conserved in the transporters of the 2-HCT family and located in the C-terminal putative TMS XI (Figure 1). The involvement of Arg425 in the interaction with the "second" carboxylate present in divalent substrates was investigated by constructing site directed mutants of the citrate transporter CitP of *Lc. mesenteroides*. Arg425 was replaced with Lys, a conservative mutation that retains the positive charge, and with the neutral Cys residue. Mutant and wild type transporters were N-terminally tagged with 10 histidines and expressed in *L. lactis* NZ9000 cells using the inducible *nisA* promoter system. The expression levels in the membrane were analyzed by immunoblotting using antibodies directed against the His-tag. The apparent molecular mass of the proteins was about 40 kDa and, under the same induction conditions, the expression levels were similar for CitP and both mutants (Figure 2).

Exchange provides a sensitive assay for the activity of CitP (7). Mutant transporters were assayed for homologous exchange in right-side-out membrane vesicles, using the high affinity substrate (S)-malate. Vesicles loaded with 5 mM radiolabeled (S)-malate were diluted 100-fold into buffer. In the absence of external substrate, efflux of (S)-malate from the membranes down the concentration gradient was a slow process for both wild type and mutant transporters (Figure 3, closed circles). Only when measured for a prolonged period of time (Figure 3C) significant efflux could be observed. The rate of efflux seemed not significantly different for the wild type and mutant transporters. Dilution of the membranes containing the wild type transporter in buffer containing 5 mM unlabeled (S)-malate resulted in very rapid release of the label from the membranes due to CitP mediated exchange (Figure 3A, closed squares). Exchange catalyzed by the Arg425Lys and Arg425Cys mutant was considerably slower, but significantly faster than efflux (Figure 3B,C, closed squares). The Arg to Cys mutation had a more drastic effect on the exchange rate than the conservative Arg to Lys mutation.

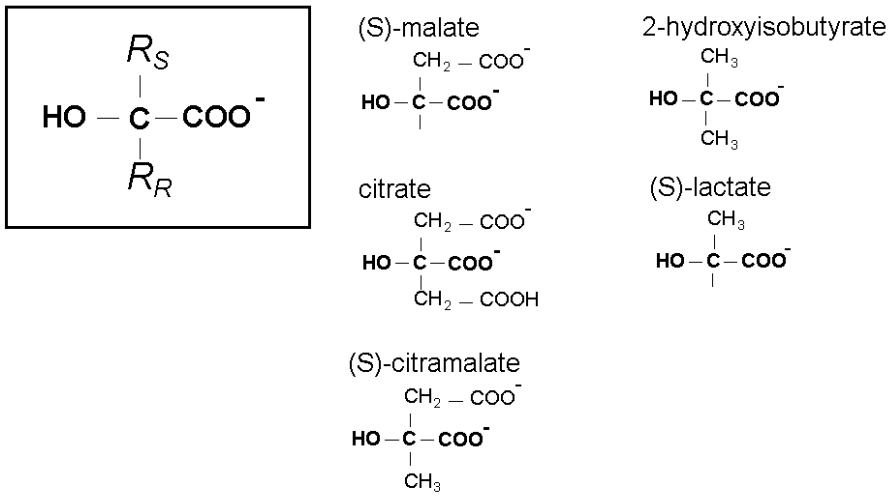

Figure 4. **CitP substrates.** Chemical structure of the substrates of CitP used in this study. The R groups are defined as R_R and R_S to discriminate between the different positions of the R groups in chiral compounds. Inset: The 2-hydroxycarboxylate motif.

Affinity for (S)-malate and 2-HIB. The kinetic characteristics of the mutant transporters were analyzed with 2-HIB and (S)-malate, a mono- and a divalent substrate of CitP, respectively (see Figure 4). Under standard induction conditions, exchange catalyzed by wild type CitP was so fast that initial rates of transport could not be measured (Figure 3A). To allow the analysis, CitP expression levels were reduced by varying the inducer concentration in the growth medium (see Experimental procedures).

The K_m^{app} for external (S)-malate at an internal concentration of 5 mM (S)-malate was 90 μM for wild type CitP in fair agreement with previous reports (6,8). In heterologous exchange using the same concentration of internal (S)-malate, the K_m^{app} for external 2-HIB was 5 mM (Figure 5A, Table 1). Replacing Arg425 by Lys, thus conserving the positive charge at position 425, reduced the affinity for (S)-malate about 10-fold (Figure 5B and Table 1). Substitution for the neutral Cys residue had a much more dramatic effect on affinity. No saturation was observed up to a concentration of 10 mM (Figure 5C). In contrast to the decrease in the affinity for divalent (S)-malate, the Arg425Lys and Arg425Cys mutants revealed an increased apparent

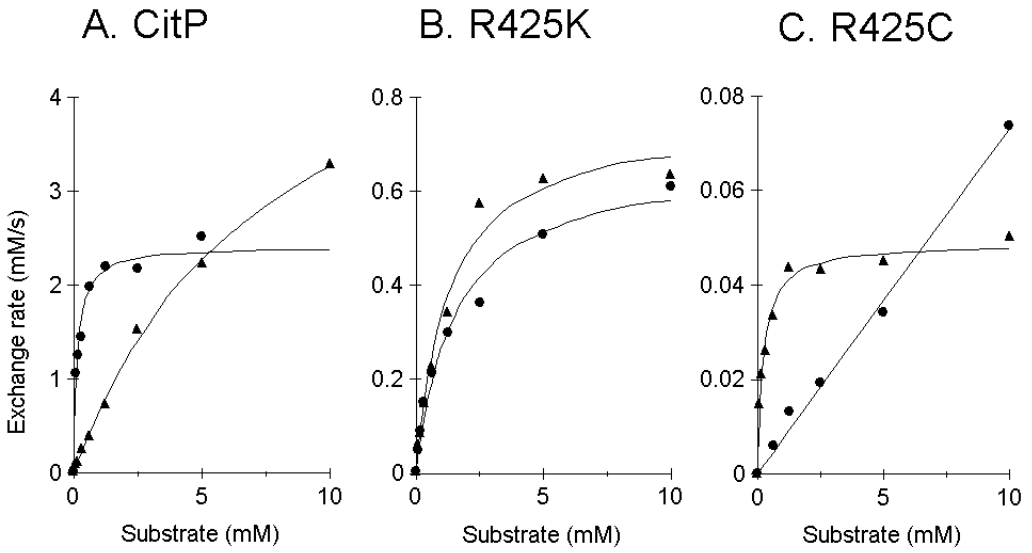

Figure 5. **Affinity of CitP (A), Arg425Lys (B), and Arg425Cys (C) for (S)-Malate and 2-HIB.** RSO membrane vesicles prepared of *L. lactis* NZ9000 expressing CitP (A), Arg425Lys (B) or Arg425Cys (C) were preloaded with 5 mM (S)-[^{14}C]malate and diluted 100-fold into buffer containing the indicated concentrations of (S)-malate (closed circles) or 2-HIB (triangles). The curves were analyzed as described in the Methods section and the obtained K_m^{app} and V_{max} values are given in Table 1. Expression level for CitP was lower than for Arg425Lys and Arg245Cys (see text).

Table1. **Kinetic parameters for CitP, Arg425Lys and R425Cys in exchange**[a]

	(S)-malate		2-HIB	
	K_m^{app} (mM)	V_{max} (mM/s)	K_m^{app} (mM)	V_{max} (mM/s)
CitP[c]	0.09 ± 0.02	2.4 ± 0.3	4.9 ± 0.6	5.8 ± 0.8
Arg425Lys	1.4 ± 0.3	0.67 ± 0.08	1.2 ± 0.3	0.76 ± 0.1
Arg425Cys	> 10[b]	> 0.07[b]	0.22 ± 0.05	0.049 ± 0.004

[a] Values were evaluated from the data shown in Figure 5 as described in the Experimental procedures section. [b] Lower limit; no significant saturation was observed. [c] Expression level for CitP was lower than for Arg425Lys and Arg425Cys (see text).

affinity for monovalent 2-HIB. The affinity of Arg425Lys increased 4-fold while Arg425Cys had a 20-fold higher affinity for 2-HIB compared to wild type CitP. The maximal rates of exchange decreased in the order CitP>Arg425Lys>Arg425Cys for both (S)-malate and 2-HIB (Figure 5 and Table 1). The results suggest that Arg425 is specifically involved in the high affinity towards the divalent (S)-malate. The positive charge of the Arg seems to play an important role in the interactions since the changes in (S)-malate affinity were much less pronounced when Arg425 was replaced with the positively charged Lys residue.

Reactivation of the Arg425Cys mutant. The methanethiosulfonate (MTS) derivatives MTSEA, MTSES and MTSET are small, charged, water soluble and cysteine specific reagents (20). They form a mixed disulfide with the thiol of the cysteine via the addition of $-SCH_2CH_2X$ groups where X is SO_3^-, $N(CH3)_3^+$, or NH_3^+ for MTSES, MTSET and MTSEA, respectively. Right-side-out membrane vesicles containing the transporters were treated with the MTS reagents and (S)-malate exchange was measured using non-saturating external (S)-malate concentrations (21). Wild type CitP contains one Cys residue at position 361 in putative TMS X. Treatment of RSO membranes containing CitP with the MTS reagents had no effect on the exchange activity (Table 2). Apparently Cys361 is not accessible to the reagents or the modification does not affect activity. In contrast, exchange catalyzed by the Arg425Cys mutant was severely affected indicating a specific modification of the Cys at position 425 (Table 2). The most striking effect was seen upon incubation with MTSEA which restores a positive charge at position 425 (Figure 6). The homologous exchange rate increased about 50-fold under the conditions of the experiment. In fact, the exchange catalyzed by MTSEA treated

Table2. **Exchange (% of control) catalyzed by Arg425Cys and CitP after treatment with sulfhydral reagents**[a].

	Arg425Cys		CitP	
	10 min	150 min	10 min	150 min
no addition	100	100	100	100
MTSEA	4800	-	100	-
MTSET	100	49	-	105
MTSES	98	40	-	110
PCMB	2	-	103	-
PCMBS	20	2	95	-

[a] RSO membrane vesicles containing Arg425Cys or CitP were treated with 1 mM of the reagents for the indicated times. After washing away the reagents vesicles were preloaded with 5mM (S)-[^{14}C]malate and diluted 100-fold into buffer containing 5 mM (S)-malate. Rates were given as the percentage of exchange by the untreated membranes.

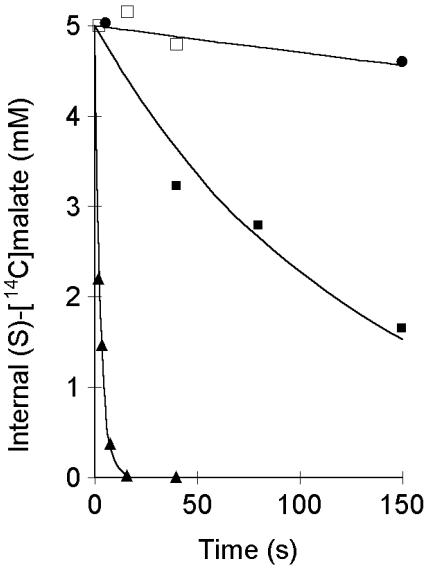

Figure 6. **Modification of Arg425Cys by MTSEA.** RSO membrane vesicles prepared of *L. lactis* NZ9000 expressing Arg425Cys were incubated for10 min, in the presence (open squares and triangles) or absence (closed circles and closed squares) of 1 mM MTSEA. After washing, the membranes were preloaded with 5 mM (S)-[^{14}C]malate and diluted 100-fold into buffer containing 5 mM (S)-malate (triangles and closed squares) or no additions (open squares and closed circles).

Arg425Cys was faster than observed for the Arg425Lys mutant (compare Figures 6 and 3B). In contrast to MTSEA, the more bulky positively charged reagent MTSET and the negatively charged MTSES reduced the (S)-malate transport activity of the mutant to undetectable levels. The results indicate that the introduction of a small positive group at position 425 in the Arg425Cys mutant can largely restore exchange activity.

Chemical modification of Arg425Lys by TNBS. TNBS reacts specifically with lysine residues and/or the N-terminus of proteins (22,23) introducing a covalently linked trinitrophenyl group in the protein. Increased reactivity at higher pH values is characteristic for a specific reaction with an amino group (23). Treatment of right-side-out membrane vesicles containing Arg425Lys with 5mM TNBS at pH 6 did not reduce the exchange activity of the transporter. However, the same treatment at pH values of 7 and 8 resulted in the loss of 35 and 80% exchange activity, respectively (Figure 7A). Under the same reaction conditions TNBS had no effect on wild type CitP (Figure 7B) indicating a specific modification of the lysine at position 425 in the Arg425Lys mutant. Apparently, modification of Lys425 in the Arg425Lys mutant renders an inactive transporter.

Accessibility of position 425. The localization of the Cys residue at position 425 in the

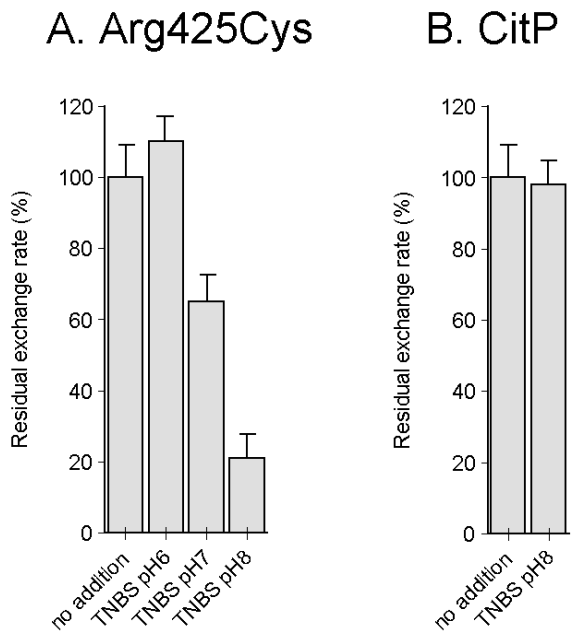

Figure 7. **TNBS treatment of Arg425Lys (A) and CitP (B).** RSO membrane vesicles prepared of *L. lactis* NZ9000 expressing Arg425Lys and CitP were incubated for 2 h with 5 mM TNBS at the indicated pH. After washing, the vesicles were loaded with 5 mM (S)-[^{14}C]malate and diluted 100-fold into buffer containing unsaturating (S)-malate concentrations i.e. 1.5 mM (S)-malate in case of Arg425Cys and 0.15 mM in case of CitP. Exchange rates were given as a percentage of the exchange rate in the untreated membranes. Error bars represent the standard deviation.

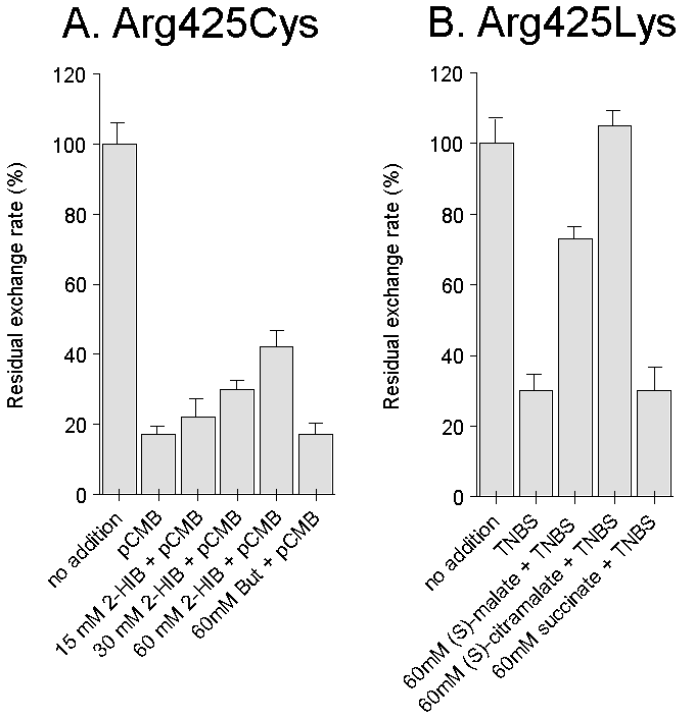

Figure 8. **Substrate protection against modification by PCMB (A) and TNBS (B).** A. RSO membrane vesicles containing Arg425Cys were incubated for 0.5 min with 0.1 mM PCMB in the presence or absence of the indicated concentrations of 2-HIB or butyrate. After washing, the membranes were preloaded with 5 mM (S)-[^{14}C]malate and diluted 100-fold into buffer containing 5 mM (S)-malate. B. RSO membrane vesicles containing Arg425Lys were incubated for 2 h with 30 mM TNBS at pH 7 in the presence or absence of 60mM (S)-malate, (S)-citramalate or succinate. After washing, the vesicles were preloaded with 5 mM (S)-[^{14}C]malate and diluted 100- fold into buffer containing 1.5 mM (S)-malate. Exchange rates were given as a percentage of the exchange rate without addition of TNBS. Error bars represent the standard deviation.

Arg425Cys mutant with respect to the membrane was investigated by the reactivity with membrane permeable and impermeable thiol reagents. MTSET is a strong base and MTSES a strong acid and both are generally regarded to be poorly membrane permeable. On the other hand, MTSEA is a weak base that equilibrates across the membrane in its undissociated form (24). Chemical modification of the Arg425Cys mutant by membrane impermeable MTSES and MTSET was incomplete even after long incubation times (Table 2). The incomplete inactivation was due to low inactivation rates as higher reagent concentrations increased inactivation (not shown). In contrast, the time course of activation with membrane permeable MTSEA was much shorter. The stimulatory effect was saturated within 10 minutes.

PCMB and its sulfonic acid derivative PCMBS are organomercurial reagents that react with high specificity with cysteine residues. PCMB is membrane permeable, while PCMBS is not. Treatment of right-side-out membrane vesicles containing Arg425Cys with either PCMB or PCMBS eventually resulted in complete inhibition of homologous (S)-malate exchange (Table 2). The same treatment had no effect on exchange catalyzed by the wild type transporter, indicating that the Cys at position 425 was modified. Inactivation of the Arg425Cys mutant by PCMBS was much slower than observed for of PCMB which fully inactivated the transporter within 3 min (Table 2). In fact, half a minute incubation with 0.1 mM PCMB resulted in 80% inhibition (see below), while the same level of inhibition by PCMBS required 10 min incubation at a 10 fold higher concentration. Taken together these results indicate that Cys425 reacts much faster with membrane permeable reagents, suggesting that the residue is accessible at the cytoplasmic side of the membrane.

Substrate protection against chemical modification. The Arg425Cys mutant revealed a relatively high affinity for external 2-HIB in heterologous exchange (K_m^{app} = 0.22 mM; Table 2) and, therefore, this substrate was selected to see whether the presence of substrate could protect the mutant against inactivation by PCMB. Right-side-out membrane vesicles were incubated with 2-HIB to equilibrate the substrate over the membrane, followed by treatment with PCMB and, subsequent removal of the substrate and unreacted PCMB (see "Experimental procedures"). In the absence of 2-HIB, the treatment resulted in 80% inhibition of exchange activity (Figure 8A). 2-HIB protected Arg425Cys against PCMB inactivation in a concentration dependent manner, but much higher concentrations were required than anticipated. Protection of Arg425Cys by 2-HIB was incomplete even at 60mM which is far

above the K_m^{app} for external 2-HIB. In the control experiment, 2-HIB was replaced by butyrate, which is very similar to 2-HIB but lacks the hydroxyl group and therefore is not a substrate of CitP (7). The presence of butyrate did not affect the inactivation by PCMB (Figure 8A), indicating that the protection by 2-HIB is specific.

(S)-Malate and another high affinity substrate of CitP (S)-citramalate (6) were tested for their potency to protect the Arg425Lys mutant against inactivation by TNBS (Figure 8B). In the presence of 60 mM (S)-malate, the 70% inhibition observed in the absence of substrate was reduced to 30% while 60 mM (S)-citramalate completely protected against inactivation under the conditions of the experiment. Similarly as observed for 2-HIB above, much higher concentrations of the substrates were required than would be expected based on the affinity constants for external (S)-malate in the homologous exchange reaction. In the control experiment, succinate which lacks the 2-hydroxy group of (S)-malate, and therefore is not a substrate of CitP, had no effect on the inhibition by TNBS.

DISCUSSION

In this study, the mechanism by which CitP transports both divalent and monovalent substrates, which is crucial to the physiological function of membrane potential generation by the transporter, was investigated. Previous substrate specificity studies showed that CitP is specific for substrates containing a 2-hydroxycarboxylate motif, $HO\text{-}CR_2\text{-}COO^-$, in which the R groups can vary. These R groups were defined as R_R and R_S to discriminate between different positions of the R groups around the asymmetric C2 atom in chiral substrates (Figure 4, inset). CitP is known to have a high affinity for substrates that have a "second" charged carboxylate at the R_S position, like citrate and (S)-malate (6). Monovalent substrates that lack this "second" carboxylate, like lactate and 2-HIB, were found to bind with low affinity but were still transported efficiently. A non-essential interaction between the protein and the "second" carboxylate of the divalent substrates was postulated which would increase affinity for these substrates. Arg425 was selected to be a good candidate for the site on the protein involved in this interaction because, (i) Arg425 is located in the stretch of 46 residues at the C-terminus, that has been suggested to be involved in the interaction with the R groups (8), (ii) Arg425 is located in a transmembrane segment of the transporter and (iii) Arg425 is conserved in the 2-HCT family (8).

It is concluded that Arg425 indeed interacts with the "second" carboxylate of the divalent substrates, based on the following observations. One, mutation of Arg425 decreased the affinity for divalent (S)-malate, but not for monovalent 2-HIB. Two, Arg425 is not essential for transporter activity. Three, a positive charge at position 425 resulted in improved transport activity. Four, chemical modification of the residue at position 425 significantly affected transporter activity. And, finally, five, the presence of substrate protected the residue at position 425 against chemical modification. The combination of the results strongly suggests that Arg425 is in the substrate binding pocket where it interacts with the "second" carboxylate. The involvement of TMS XI in the binding site is in line with the behaviour of chimeric transporters (8).

The affinities of the mutant and wild type transporters were determined by homologous and heterologous exchange using membrane vesicles loaded with a fixed (S)-malate concentration and varying the external substrate concentrations. The determined affinities are for the external substrate. In a ping-pong type exchange mechanism the apparent affinity for the external substrate is dependent on the affinity and concentration of the internal substrate. Non-saturating conditions at the inside result in higher apparent affinities for the external substrate and lower maximal rates. Consequently, a mutation that lowers the affinity for internal (S)-malate decreases the K_m^{app} for external substrate and the maximal rate of exchange. The kinetics observed with 2-HIB of the Arg425Lys and Arg425Cys mutants are consistent with a decrease in internal (S)-malate affinity in the order Arg425, Lys425, Cys425, if the binding affinity for 2-HIB would not change significantly. The kinetic affinity for external 2-HIB

increases and the maximal rate for heterologous 2-HIB/(S)-malate, as well as for homologous (S)-malate exchange, decreases (Figure 5). The observed decreased affinity of the mutants for external (S)-malate in homologous exchange strongly argues in favour of a decreased binding affinity of the protein for (S)-malate with a subsequent lowering of the kinetic affinity for internal (S)-malate.

The guanidinium group of the Arg425 residue is not absolutely essential for transport function since even a non-conservative substitution for Cys does not abolish the activity of CitP. However, the affinity for divalent (S)-malate was severely affected by this mutation. The drop in affinity was less when Arg425 was mutated to Lys, and, especially, the reactivation of Arg425Cys by MTSEA, which restores a positive charge at position 425, strongly suggest that the positive charge is essential for high affinity binding of divalent substrates. Nevertheless, the positive charge is not the only relevant factor since Arg425 cannot be replaced with lysine or MTSEA labelled cysteine without some loss of affinity. The chemistry of the guanidinium group allows for the formation of two hydrogen bonds which is known to result in an interaction of unusual strength with a carboxylate group (25). Alternatively, only an Arg residue can position its charge accurately relatively to the substrate for an optimal interaction. In contrast to the stimulation of exchange by MTSEA, positively charged MTSET inhibited the Arg425Cys mutant. The three methyl groups of the quaternary ammonium group of the reagent are likely to interfere sterically with substrate binding and thus inactivate the transporter. MTSES, PCMB, and PCMBS inactivated Arg425Cys and TNBS Arg425Lys most likely because of the presence of a negative charge on the side chains that is expected to repel the carboxylate of the substrate and/or by steric interference with the substrate.

In the topology model of CitP, Arg425 is located at the cytoplasmic side of TMS XI (9-11). The present data supports this localisation. The chemical inactivation of the Arg425Cys mutant in right-side-out vesicles was much faster for the membrane permeable PCMB than for its impermeable counterpart pCMBS (Table 2). Since the two probes have similar chemical reactivities, the difference in inactivation rate suggests that the reactive cysteine is only accessible from the inside. Similarly, chemical modification by the membrane impermeable MTSET and MTSES was very slow while modification by the permeable MTSEA was fast. These low reaction rates are not due to differences in chemical reactivity with the thiol groups. In fact, MTSET is known to have 2.5 fold higher intrinsic reactivity for sulfhydrals than MTSEA (26). Thus, also these results suggest that the reaction takes place at the internal face of the membrane. The low rate of modification with PCMBS, MTSES and MTSET may be explained by a slow permeation through the membrane by these reagents followed by reaction at the cytoplasmic side or by a low accessibility of the Cys residue from the outside. Accessibility of a binding site residue from either side of the membrane is not unlikely since the transporter has to expose the binding site alternately to the two sides of the membrane during turnover.

The presence of substrate protected against chemical modification of the residue at position 425 which is consistent with the assignment of Arg425 as a binding site residue. Interestingly, 2-HIB protected Arg425Cys against PCMB inactivation in a concentration dependent manner, but protection was still incomplete at a concentrations of 60 mM, indicating a very low affinity for the substrate. This contrasted with the high apparent affinity for external 2-HIB in the exchange assays, K_m^{app} = 0.22 mM (Table 1). The same situation was observed for the protection of Arg425Lys by (S)-malate against inhibition by TNBS. Possible explanations for the apparent discrepancy may be the underestimation of the binding affinity for external substrate when deduced from a kinetic experiment as discussed above or, alternately, the transporter has a much lower affinity for internal substrate than for external substrate. In any case, full protection against chemical modification was shown to be possible by the high affinity substrate (S)-citramalate.

In conclusion, the combination of the kinetic characteristics of the mutants and the chemical modification and substrate protection against chemical modification strongly suggests a direct

interaction between Arg425 and the carboxylate at the R_S groups of the divalent substrates. The positive charge of Arg425 seems to be important in this interaction but the chemistry of the guanidinium groups may play a role as well. Although Arg425 is not essential for transport by CitP, its role in specifically interacting with the "second" carboxylate of di- and tricarboxylate substrates makes it essential for the generation of the membrane potential which is the physiological function of CitP.

REFERENCES

1. Lolkema, J. S., Poolman, B., and Konings, W. N. (1996) In Konings, W. N., Kaback, H. R., and Lolkema, J. S., editors. *Handbook of Biological Physics*, Elsevier, Amsterdam
2. Bandell, M., Lhotte, M. E., Marty Teysset, C., Veyrat, A., Prevost, H., Dartois, V., Divies, C., Konings, W. N., and Lolkema, J. S. (1998) *Appl.Environ.Microbiol.* **64,** 1594-1600
3. Marty Teysset, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1995) *J.Biol.Chem.* **270,** 25370-25376
4. Marty Teysset, C., Posthuma, C., Lolkema, J. S., Schmitt, P., Divies, C., and Konings, W. N. (1996) *J.Bacteriol.* **178,** 2178-2185
5. Magni, C., de Mendoza, D., Konings, W. N., and Lolkema, J. S. (1999) *J.Bacteriol.* **181,** 1451-1457
6. Bandell, M. and Lolkema, J. S. (1999) *Biochemistry* **38,** 10352-10360
7. Bandell, M., Ansanay, V., Rachidi, N., Dequin, S., and Lolkema, J. S. (1997) *J.Biol.Chem.* **272,** 18140-18146
8. Bandell, M. and Lolkema, J. S. (2000) *Biochemistry* **39**, 13059-13067
9. van Geest, M. and Lolkema, J. S. (1996) *J.Biol.Chem.* **271,** 25582-25589
10. van Geest, M., Nilsson, I., von Heijne, G., and Lolkema, J. S. (1999) *J.Biol.Chem.* **274,** 2816-2823
11. van Geest, M. and Lolkema, J. S. (1999) *J.Biol.Chem.* **274,** 29705-29711
12. De Ruyter, P.-G.-G.-A., Kuipers, O.-P., and De Vos, W.-M. (1996) *Applied and Environmental Microbiology* **62,** 3662-3667
13. de Ruyter, P. G., Kuipers, O. P., and De Vos, W. M. (1996) *Appl.Environ.Microbiol.* **62,** 3662-3667
14. Kuipers, O. P., Beerthuyzen, M. M., Siezen, R. J., and De Vos, W. M. (1993) *Eur.J.Biochem.* **216,** 281-291
15. Putman, M., van Veen, H. W., Poolman, B., and Konings, W. N. (1999) *Biochemistry* **38,** 1002-1008
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2 Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K., and Pease, L. R. (1989) *Gene* **77,** 61-68
18. Holo, H. and Nes, I. F. (1989) *Appl.Environ.Microbiol.* **55,** 3119-3123
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J.Biol.Chem.* **193,** 265-275
20. Akabas, M. H., Stauffer, D. A., Xu, M., and Karlin, A. (1992) *Science* **258,** 307-310
21. Ramamoorthy, S., Melikian, H. E., Qian, Y., and Blakely, R. D. (1998) *Methods Enzymol.* **296,** 347-370
22. Fields, R. (1972) *Methods in Enzymology* **25,** 464-468
23. Grubmeyer, C., Segura, E., and Dorfman, R. (1993) *J.Biol.Chem.* **268,** 20299-20304
24. Olami, Y., Rimon, A., Gerchman, Y., Rothman, A., and Padan, E. (1997) *J.Biol.Chem.* **272,** 1761-1768
25. Mitchell, J. B., Thornton, J. M., Singh, J., and Price, S. L. (1992) *J.Mol.Biol.* **226,** 251-262
26. Karlin, A. and Akabas, M. H. (1998) *Methods Enzymol.* **293,** 123-145

Summary

RESEARCH OBJECTIVE

Most secondary transporters require the metabolic energy stored in electrochemical H^+ or Na^+ gradients across the cell membrane to drive transport of their substrates. However, in some cases substrate is taken up via the transporter in exchange for the metabolic breakdown product of the substrate which accumulates intracellularly. This is termed precursor-product exchange. The citrate transporter of *Leuconostoc mesenteroides* (CitP) and the malate transporter of *Lactococcus lactis* (MleP) both function as precursor-product exchangers. CitP and MleP are able to exchange respectively external citrate or (S)-malate for internal lactate, the metabolic end product of citrate and (S)-malate degradation in lactic acid bacteria. The uptake of the external substrate is driven by the chemical concentration gradients of the internal product and the external substrate. This generates a membrane potential since the exchange between the divalent citrate or (S)-malate and the monovalent lactate is electrogenic. Thus exchange generates metabolic energy for the cell. The ability of CitP and MleP to transport substrates that differ in structure and charge implies specific requirements for the substrate binding site. The object of the research described here was to identify protein-substrate interactions that provide affinity and specificity for CitP and MleP and more specific, that provide CitP and MleP the ability to transport substrates that differ in charge and structure. As long as a method for high resolution structure determination of secondary transporters is not available structural-function relationships must be obtained from biochemical and biophysical experiments.

SUBSTRATE SPECIFICITY STUDIES INDICATE HYDROPHOBIC AND ELECTROSTATIC INTERACTIONS WITH THE SUBSTRATE.

The genes coding for CitP and MleP were cloned and characterised in a citrate and malate transport negative *L. lactis* strain. Functional analysis of the *citP* and *mleP* gene products revealed the same properties as observed in membrane vesicles of *Lc. mesenteroides* and the malate transport positive *L. lactis*. CitP and MleP were shown to be homologous. The protein family to which they belong includes the citrate transporter of *Klebsiella pneumoniae* (CitS) which is not involved in precursor-product exchange. MleP, CitP and CitS were analysed for their substrate specificity. A 2-hydroxycarboxylate motif common to all substrates, i.e. HO-CR_2-COO^- was found to be essential for transport although also some 2-oxocarboxylate and 3-hydroxycarboxylates were transported to a low extent. Any modification of the OH or COO^- groups drastically reduced the affinity of the transporters for the substrates, indicating their relevance in substrate recognition.

Substrate specificity differences between the transporters were found to be due to differences in tolerance towards the two R substituents at the C2 atom. CitS was found to have a narrow substrate specificity transporting mainly citrate and to a low extent citramalate. In contrast, the precursor-product exchangers CitP and MleP have a low specificity for the R groups. In addition to their physiological substrates they transport a wide range of 2-hydroxycarboxylates with R groups ranging from two hydrogen atoms (glycolate) to a phenyl group (mandelate). The tolerance towards the R groups allows these transporters to accept substrates that differ in charge and structure of the R groups and thus act as precursor-product exchangers.

The broad specificity of CitP and MleP towards the R groups allowed an analysis of the effects that different R groups have on affinity and translocation efficiency. The R groups were defined as R_R and R_S to discriminate between the different positions of an R group in chiral substrates. The affinity of CitP for divalent substrates such as citrate, (S)-citramalate and (S)-malate that have a charged carboxylate at the R_S position was at least one order of magnitude higher than for monovalent substrates that lack this carboxylate, such as (S)-lactate, (R)-lactate

and 2-hydoxyisobutyrate. A similar substrate specificity was found for MleP. The high affinity of MleP and CitP for substrates containing a "second" carboxylate at the R_S position was explained by postulating a strong, possibly electrostatic, interaction between the proteins and the "second" carboxylate that is responsible for the high affinity binding. Divalent substrates that have a charged carboxylate at the R_R position, such as (R)-malate and (R)-citramalate were not bound by the transporters indicating that the interaction was confined to the R_S position. The affinity for glycolate in CitP and MleP was found to be lower than their respective affinities for (S)- or (R)-lactate or hydroxyisobutyrate. Glycolate differs from the latter substrates in that it has H atoms instead of CH_3 groups at the R_R or R_S position. The difference in affinity between the substrates was explained by postulating an interaction of MleP and CitP with hydrophobic R_R and R_S groups that promotes affinity. A hydrophobic interaction was speculated. Overall substrate specificity of CitP and MleP is similar. The two main differences depend on subtle differences in the interactions with the R groups. Firstly, while CitP is able to bind and translocate substrates with large R_R groups, MleP is only binds such compounds. This explains MlePs inability to translocate citrate which also has a large R_R group. Secondly, CitP has a very low affinity for substrates with bulky R_S groups while MleP binds these substrate groups with moderate affinity.

CHIMERAS IDENTIFY THE CONSERVED C-TERMINUS TO BE INVOLVED IN SUBSTRATE SPECIFICTY

The differences in substrate specificity between MleP and CitP were exploited to identify residues that may be part of the substrate binding site and the translocation pore. The conserved C-terminal region of the transporters was investigated for its role in substrate recognition by constructing chimeric transporters. An advantage of this approach is that subtle differences in substrate specificity between CitP and MleP may depend on a combination of different amino acid residues at multiple positions which may be difficult to trace using site directed mutagenesis. Interchanging the C-terminal 46 residues between MleP and CitP had no effect on the affinity for divalent substrates such a citrate and (S)-malate. However, it significantly affected the affinity for a number of monovalent substrates with bulky R_R or R_S groups in a complementary manner. The resulting substrate specificity of the chimeras mimicked to a certain extent the donor of the C-terminus. It was concluded that the conserved C-terminus determines, at least in part, substrate specificity by interacting with neutral R_R and R_S groups. The results also revealed that the effects of interchanging the C-terminus were different for different monovalent substrates. This indicated that different neutral R groups interact with the protein via different interactions. The observation that the chimeras did not completely adopt specificity of the donor of the C-terminus indicated that not all residues interacting with the R groups are located in the C-terminus.

SITE DIRECTED MUTAGESIS IDENTIFIES ARG425 TO BE INVOLVED IN AFFINITY TOWARDS DIVALENT SUBSTRATES

Site directed mutagenesis of CitP indicated that the conserved Arg425, located in putative TMS XI, is involved in the electrostatic interaction with the charged carboxylate of divalent substrates. Mutagenesis of Arg425 resulted in a decrease in affinity for divalent substrates but not for monovalent substrates. Moreover, chemical modification of residues at this position was blocked by the presence of substrate. Interestingly, the topology model of the 2-HCT transporters places the Arg425 at the cytoplasmic face of the membrane. Accessibility studies in membrane vesicles using membrane permeable and impermeable chemical reagents indicated that indeed position 425 is best accessible from the inside of right side out membrane vesicles.

CONCLUDING REMARKS

A combination of substrate specificity studies, analysis of chimeras and analysis of site directed mutants allowed us to propose a model of the substrate binding site (Chapter 1). A charged residue (Arg425) interacts with substrates with a charged carboxylate at the R_S position while the hydrophobic surrounding of Arg425 interacts with neutral R groups. Arg425 is thought to reside close to the cytoplasmic face of the membrane. This raises the question of how a hydrophilic substrate can access a substrate binding site which is apparently buried in a hydrophobic environment. Possibly a somewhat hydrophilic cleft in the protein lines the route towards the substrate binding site.

The ability to translocate substrates that differ in structure (and charge) is a property of only few transporters. Most well known are the mitochondrial exchangers, the bacterial precursor-product exchangers and the multi drug transporters. The mechanisms behind the promiscuity towards the substrates may be similar. Some studies on some multi drug transporters identified negatively charged residues that interacted specifically with positive charges on the substrate. Moreover, neutral substrates are thought to interact with the protein via a set of interactions that differ per substrate. These properties are similar to what was found for the interaction of CitP and MleP with the R groups of the substrate.

The model of the substrate binding site of CitP and MleP contains a number of postulated interactions between the substrate and the protein for which yet the responsible amino acid residues have to be identified. The identified interaction sites, topology, substrate specificity and sequence conservation allow an educated guess as to what residues are likely to be involved in the substrate-protein interactions.

Samenvatting

Een bacterie is een ééncellig organisme. De bacteriële cel wordt omgeven door een celmembraan. De celmembraan vormt een laag om de gehele cel, die ondoorlaatbaar is voor water en voor daarin opgeloste stoffen. Zodoende houdt de cel zijn inhoud, met daarin o.a. het DNA en een scala aan eiwitten, gescheiden van zijn omgeving en voorkomt dat ongewenste stoffen de cel binnen komen. Om te leven heeft een bacterie echter voedingsstoffen uit zijn omgeving nodig. Om het opnemen van deze voedingsstoffen mogelijk te maken bevinden er zich in de celmembraan een groot aantal eiwitten. Voedingsstoffen worden door deze zogenaamde transporteiwitten gebonden waarna ze op een gecontroleerde manier door de celmembraan heen worden getransporteerd. De moleculaire mechanismen achter deze transportprocessen zijn nog grotendeels onbekend.

Bacteriën kunnen leven op een zeer groot aantal voedingstoffen die door nagenoeg evenzoveel transporteiwitten worden opgenomen. De bacteriën *Leuconostoc mesenteroides* en *Lactococcus lactis* voeden zich onder andere met citroenzuur (citraat) en appelzuur (malaat). De citraat- en malaatmoleculen worden opgenomen met behulp van een citraat-transporteiwit (CitP genaamd) en een malaat-transporteiwit (MleP genaamd). Tegelijk met de opname van deze stoffen zorgen deze transporteiwitten voor een uitscheiding van melkzuur (lactaat), het eindproduct van citraat- en malaatafbraak in de cel. Ons onderzoek heeft zich gericht op de analyse van het moleculaire mechanisme waarmee de genoemde transporteiwitten citraat, malaat, of lactaat herkennen en door de celmembraan heen transporteren. Door middel van genetische en biochemische experimenten werd inzicht verkregen in de moleculaire interacties tussen de getransporteerde stoffen en de transporteiwitten.

Om te beginnen werden de twee genen die verantwoordelijk zijn voor de aanmaak van CitP en MleP geanalyseerd (Hoofdstuk 2 en 3). Er werd gevonden dat de genen voor zowel CitP als MleP sterk overeenkomen met het gen voor een transporteiwit uit de bacterie *Klebsiella pneumoniae* (CitS genaamd). Hieruit kon worden afgeleid dat CitP en MleP qua architectuur sterk op CitS lijken. Over de architectuur van CitS was al het nodige bekend en deze kennis leverde nuttige informatie voor CitP en MleP.

Vervolgens werd onderzocht of CitP en MleP, naast citraat, malaat en lactaat, nog andere stoffen door de membraan heen kunnen transporteren (Hoofdstuk 3 + 4). CitP en MleP bleken een groot aantal andere stoffen te kunnen transporteren. Alle getransporteerde stoffen (substraten) hebben een opmerkelijke overeenkomst wat betreft hun moleculaire structuur. Zij hebben de structuurformule $HO\text{-}CR_2\text{-}COO^-$ (het 2-hydroxycarboxylaat motief), waarin de twee R-groepen variabel zijn. Stoffen die niet deze structuur hebben worden slecht of helemaal niet getransporteerd door deze eiwitten. De R groepen in dit motief kunnen variëren van waterstofatomen tot een benzeenring. Zowel CitP en MleP hebben echter een duidelijke voorkeur voor een negatief geladen carboxylaat als R groep. Substraten die een dergelijke carboxylaat groep bezitten worden veel sterker gebonden dan substraten die deze groep niet hebben, zoals lactaat. Dit wees erop dat CitP en MleP een positief geladen aminozuur (aminozuren zijn de bouwstenen van eiwitten) bezitten waarmee zij de negatief geladen carboxylaat groep aantrekken.

Naast bovengenoemde overeenkomsten werden ook enkele verschillen tussen CitP en MleP gevonden. Eén van de verschillen is dat MleP substraten met grote neutrale R groepen veel beter bindt dan CitP. Om erachter te komen welk deel van de eiwitten verantwoordelijk is voor dit verschil werden, m.b.v. genetische technieken, zogenaamde chimere eiwitten geconstrueerd (Hoofdstuk 5). Chimere eiwitten zijn eiwitten die kunstmatig zijn samengesteld uit delen van twee verschillende eiwitten. Een klein gedeelte van CitP, de zogenaamde C-terminus, werd vervangen door de C-terminus van MleP. Dit "chimere eiwit" functioneert en heeft met MleP gemeen dat het goed met moleculen met grote neutrale R groepen kan binden. Na vervanging

van de C-terminus van MleP door de C-terminus van CitP heeft het "chimere eiwit" met CitP gemeen dat het slecht met moleculen met grote neutrale R groepen kan binden. Hieruit is geconcludeerd dat de C-terminus bepalend is voor de goede of slechte binding met dit soort substraten en dus een interactie aangaat met dit soort substraten.

Een van de aminozuren in de C-terminus, namelijk een arginine, is positief geladen. Een dergelijk aminozuur zou de voorkeur van CitP en MleP voor substraten met een negatief geladen carboxylaat op een van de R groepen kunnen veroorzaken. Om te bepalen of dit daadwerkelijk het geval is werd het arginine in CitP, m.b.v. genetische technieken, vervangen door een neutraal aminozuur, namelijk een cysteine (Hoofdstuk 6). Hierna werden substraten met een negatief geladen carboxylaatgroep veel minder sterk gebonden door CitP terwijl substraten zonder de carboxylaatgroep even sterk of zelfs sterker gebonden werden. Hieruit kon worden afgeleid dat dit arginine inderdaad specifiek bindt aan de geladen carboxylaat op de R groep. Extra bewijs voor een interactie tussen het arginine en de substraten werd verkregen door middel van experimenten die aantoonden dat binding van een substraat aan het eiwit de binding van andere moleculen blokkeert.

Abbreviations

$\Delta\psi$	membrane potential
ΔpH	pH gradient
2-HCT	2-hydroxycarboxylate transporter
2-HIB	2-hydroxyisobutyrate
COVs	cytochrome *c* oxidase containing vesicles
MTSEA	2-aminoethyl methanethiosulfonate hydrobromide
MTSES	sodium (2-sulfonatoethyl)methanethiosulfonate
MTSET	[2-(trimethylammonium)ethyl]methanethiosulfonate bromide
OD_{660}	optical density at 660 nm
ORF	open reading frame
PCMB	*p*-chloromercuribenzoic acid
PCMBS	*p*-chloromercuribenzosulfonate
PCR	polymerase chain reaction
PMF	proton motif force
RSO (vesicles)	right-side-out (membrane vesicles)
SDS-PAGE	sodium dodecyl sulfate - poly acrylamide gel electrophoresis
TMPD	N,N,N', N'-tetramethyl-*p*-phenyleneiamine
TMS	trans membrane segment
TNBS	2,4,6-trinitrobenzene sulfonate

List of publications

Bandell, M., Hall, M.J., Wang, G., Seto Young, D., and Perlin, D.S. (1996) Probing the cytoplasmic LOOP1 domain of the yeast plasma membrane H^+-ATPase by targetted factor Xa proteolysis. *Biochimica et Biophysica Acta* **1280(1)**, 81-90.

Bandell, M., Anansay, V., Rachidi, N., Dequin, S., and Lolkema, J.S. (1997) Membrane potential-generating malate (MleP) and citrate (CitP)transporters of lactic acid bacteria. *Journal of Biological Chemistry* **272(29)**, 18140-18146.

Seto Young, D., Bandell, M., Hall, M.J., and Perlin, D.S. (1998) Differential exposure of surface epitopes in the beta strand region of LOOP1 of the yeast H^+-ATPase during catalysis. *Journal of Biological Chemistry* **273(29)**, 18282-18287.

Bandell, M., Lhotte, M.E., Marty Teysset, C., Veyrat, A., Prevost, H., Dartois, V., Divies, C., Konings, W.N., and Lolkema, J.S. (1998) Mechanism of the citrate transporters in carbohydrate and citrate cometabolism in *Lactococcus* and *Leuconostoc* species. *Applied and Environmental Microbiology* **64(5)**, 1594-1600.

Bandell M., and Lolkema, J.S. (1999) Stereoselectivity of the membrane potential-generating citrate and malate transporters of lactic acid bacteria. *Biochemistry* **38(32)**, 10352-10360.

Bandell M., and Lolkema, J.S. (2000) The conserved C-terminus of the citrate (CitP) and malate (MleP) transporters of lactic acid bacteria is involved in substrate recognition. *Biochemistry* **39(42)**, 13059-13067.

Bandell M., and Lolkema, J.S. (September 18, 2000) Arg425 of the citrate transporter CitP is responsible for high affinity binding of di- and tricarboxylates. *Journal of Biological Chemistry* 10.1074/jbc.M005940200. Published online ahead of print.

Stellingen

behorende bij het proefschrift:

Substrate Recognition by the 2-Hydroxycarboxylate Transporter Proteins

van Michael Bandell

1) De goede werkgelegenheid en hoge salarissen voor niet-gepromoveerde academici maakt promoveren een kostbare aangelegenheid.

2) In tegenstelling tot wat men logischerwijze zou verwachten is juist het leggen van schijnbaar onlogische verbanden belangrijk in de wetenschap.

3) Moleculair biologisch onderzoek vergt ruimtelijk inzicht.

4) Een onderzoeker moet niet alleen kunnen doorzetten maar ook tijdig kunnen opgeven.

5) Voor de stimulering van startende biotechnologie bedrijven in Nederland is een cultuuromslag nodig waarbij een faillissement niet langer het stigma draagt van een mislukking.

6) In een sluis komt de ware aard van de schipper naar boven.

7) Een promotie is een vrij omslachtige manier om een feest te kunnen geven.

8) Getuige de resultaten bij het penalties schieten is het Nederlands hockey elftal stressbestendiger dan het Nederlands voetbal elftal.